STANDARDIZATION AND PHARMACOLOGICAL SCREENING OF HEPATOPROTECTIVE, DIURETIC, AND ANTI - OXIDANT ACTIVITIES OF SIDDHA FORMULATION MILAGU USITHAM

In partial fulfillment of the requirements for the award of the degree of

DOCTOR OF MEDICINE (SIDDHA)

BRANCH-II-GUNAPADAM

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Chennai – 47.

DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation entitled **"Standardization and Pharmacological screening of Hepatoprotective, Diuretic, and Anti - oxidant activities of siddha formulation *Milagu Usitham"*** is a bonafide and genuine research work carried out by me under the guidance of **Dr.S.Sivakkumar M.D.,(s) Ph.D.,** Associate Professor, Department of Gunapadam, National Institute of Siddha, Chennai – 47 and the dissertation has not formed the basis for the award of any Degree, Diploma, Fellowship or other similar title.

Date: **Signature of the Candidate**

Place: Chennai- 47 **Dr. D.VELAMAN**

CERTIFICATE BY THE GUIDE

This is to certify that the dissertation entitled **"Standardization and Pharmacological screening of Hepatoprotective, Diuretic, and Anti - oxidant activities of siddha formulation *Milagu Usitham"*** is submitted to The Tamil Nadu Dr. M.G.R. Medical University in partial fulfilment of the requirements for the award of degree of M.D (Siddha) - Gunapadam is the bonafide and genuine research work done by **Dr. D.Velaman (Reg.No-321912208)** under my supervision and guidance. The dissertation has not formed the basis for the award of any Degree, Diploma, Fellowship or other similar title.

Date: **Signature of the Guide**

Place: Chennai- 47 **Dr.S.SIVAKKUMAR**

BONAFIDE CERTIFICATE

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CONTENTS

1. INTRODUCTION

Siddha system of medicine is enriched with unique and peculiar aspects with treasure house of secret science. It was created by Lord Shiva. This was taught to his disciple Agatya. After this he taught him all the Siddhars and thereby reached out to the people. This is why Agasthiyar is the forerunner of all the eighteen Siddhars. The main goal of the Siddha medicine is

- To make one mentally and physically perfect
- To make body imperishable
- To promote longevity

Their thoughts and teachings were crystallized in the form of a great system of medicine. According to *Thirumoolar*,

"One that cures physical ailment is medicine
One that cures psychological ailment is medicine
One that prevents ailment is medicine and
One that bestows immortality is medicine" (1)

Siddha medicine preparations include herbs, metals, minerals, animal byproducts. In Siddha system, there one 64 different types of medications -32 types of internal medicines and 32 types of external medications.

Chooranam is one among the 32 types of internal medicines [2] *Chooranam* are fine dry powder of drugs.The *Chooranam* should be very fine, amorphous and should be perfectly dry before the *Chooranam* preparation, ingredients has to be be purified . *Milagu Usitham* is one among the herbal formulation mentioned in the text "*Anuboga vaidya navaneetham*, Part- 9". The ingredients of *Milagu Usitham* are purified Milagu (*Piper nigrum*) and *Poovarasam pattai* (*Thespesia populnea*).Its indication for *Manjal kamalai* (Liver Disease) and *Manjalmegam* (Veneral Disease) [3].

In our OPD and IPD of Ayothidoss Pandithar Hospital attached with National Institute of Siddha , many cases of patients are reporting with liver disese . So the researcher chosen to explore the Siddha medicine *Milagu Usitham* for liver disorders.

The World liver day is observed on every 19th April, to spread awareness about liver related disease. As per the World Health Organisation's published information 2017, liver disease deaths in India reached 2,59,749 or 2.9% of total deaths. Liver disease may affect one in every 5 Indians [4].

The liver is the second largest and the most complex organ in the body. Liver has a pivotal role in regulation of physiological processes. It is involved in several vital functions such as metabolism, secretion and storage. Furthermore, detoxification of a variety of drugs and xenobiotics occurs in liver. The bile secreted by the liver has, among other things, an important role in digestion. Liver diseases are among the most serious ailment. They may be classified as acute or chronic hepatitis (inflammatory liver diseases), hepatosis (non inflammatory diseases) and cirrhosis (degenerative disorder resulting in fibrosis of the liver).

Chronic liver diseases (CLD) cause significant morbidity and mortality worldwide. Mortality data is most often used to assess the disease burden and there had been a 46% increase in CLD mortality in the world between 1980 to 2013, underscoring the emerging public health importance of CLD. Most of this increase in CLD mortality has been reported from the low and low-middle income (LMIC) countries of Asia and Africa [4]. Globally, 1.5 billion persons had CLD in 2017, most commonly resulting from NAFLD (60%), HBV (29%),HCV (9%), and ALD (2%)(4). In European countries, the median cirrhosis prevalence was 833 of 100,000 (range,447–1100) Treatment options for common liver diseases such as cirrhosis, fatty liver and chronic hepatitis are problematic. The effectiveness of treatments such as interferon, colchicine, penicillamine and corticosteroids are inconsistent at best and the incidence of side effects profound. It may also cause various other diseases. So, there is a need for safe and potent hepatoprotective drug to treat various liver disease. In recent years synthetic drugs are showing more adverse effect, to overcome this problem researchers are trying to avoid this risk of those drugs. Whenever a drug is prescribed to a patient, they are facing risk of side effect, so long-term use of these drugs patient may cause for CLD.

In herbal medicine the toxicity of herbal drugs is less when compared with the synthetic

medicines. Due to known side effect syenthitic drug patient preferred to take alternative medicine which is natural and healthy way . The process of healing and regeneration of liver cells are support and promote by herbal preparations with less side effects.

Still there is no scientific background regarding the standardization and pharmacological effect of Siddha formulation *Milagu Usitham.* Hence the reseacher has choosen the drug *Milagu Usitham* for this study to standardize and evaluate the pharmacological activities such as Hepato-protective, Diuretic and Anti- Oxitant .

2. AIM AND OBJECTIVES

Aim:

Standardization and Pharmacological screening *of* Hepatoprotective, Diuretic, *and A*nti-oxidant activities of Siddha formulation *Milagu Usitham* in *Invivo* and *Invitro* model.

Primary objective:

To screen the pharmacological activities Hepatoprotective, Diuretic and Anti-oxidant of *"Milagu Usitham"*

Secondary Objectives :

- To study the safety of *Milagu Usitham* through acute toxicity as per OECD – 423 Guideline

- To study the Standardization parameters of *Milagu Usitham* as per PLIM Guideline.

3.1.1 *Milagu* – Siddha Aspect

வேறுபெயர்கள்

கலினை , கறி, காயம், கோளகம், திரங்கல், மிரியல், சருமபந்தம், வள்ளிசம், மாசம்,குறுமிளகு, மலையாளி

சுவை

கைப்பு கார்ப்பு

வீரியம்

வெப்பம்

செய்கைகள்

- காறலுண்டாக்கி
- அகட்டுவாயுவகற்றி
- முறைவெப்பகற்றி
- தடிப்புண்டாக்கி
- வெப்பமுண்டாக்கி
- வீக்கங்கரைச்சி
- வாதமடக்கி
- நச்சரி

பொதுக்குணம்

அளவையுறாக்காரம் அடைந்திடும் வாத

விளவையல் லாமறுக்கும் மெய்யே மிளகின்காய்

கண்டவர்க்கும் இன்பமாம் காரிகையே செழ்மூலங்

கொண்டவர்க்கு நான்மறுந்தாங் கூறு[6]

இதனால் குளிர்சுரம் , பாண்டு, கோழை, கழிச்சல், குன்மம், வாயு, சுவையின்மை வெறி, மூலம், சன்னியாசம், அபஸ்மாரம், பிரமேகம், இருமல், பக்கவாதம், களநோய் குய்யரோகம், சோனி வாதம், ரத்தகுன்மம், செரியாமை, காமாலை இவை போகும்.

பழம்

சீதசுரம் பாண்டு சிலேத்மங் கிராணி குன்மம்
வாதம் அருசிபித்தம் மாமூலம் ஓதுசன்னி
யாசமபஸ் மாரம் அடன்மேகம் காசமிவை
நாசங் கறிமிளகினால்

கோணுகின்ற பக்கவலி குய்யவுரோ கம்வ
சோனிதங்க ழுத்திற்குள் தோன்றுநோய் காணரி
காதுநோய் மாதர்குன்மங் காமாலை மந்தமென்றீர்
ஏதுநோய் காயுருக்கில் ஈங்கு

மிளகின் பெருமை

தீயாகி ங்கும்யாக் திரியுமதை யாவத்து
மேயாம லெப்படியு முண்டாக்காற் பாயாது
போந்திமிர்வாத ங்கிரந்தி புண்ணீரும் மண்ணவர்க்கும்
காந்திமெய்வா தச்சலுப்பைக் காய்

மிளகு வளி, தீ, கபகுற்றங்கள் இவை அனைத்தையும் நீக்கும். அன்றியும் திமிர்வாதம், கழலை, வளி, சளி இவைகளை நீக்கும். (Figure no 1)

Synonym

Tamil	-	Milagu
English	-	Black pepper
Sanskrit	-	Maricha
Telugu	-	Miriyaluu
Hindi	-	Kali-Mirch
Malayalam	-	Kurumulaku
Kannadam	-	Menasu

Taste:

Kaippu, Kaarppu

Potency:

Heat

Pirivu:

Kaaruppu

Actions:

- Acid
- Carminative
- Antiperiodic
- Rinefacoemt
- Stimulant
- Resolvent
- Antivatha
- Antidote

Purification of *Milagu:*

Soak in the butter mil overnight then washed and dry in sunlight.

மிளகு சேரும் மருந்துகள்

காந்தாதி மண்டூர செந்தூரம்[7]

அளவு - 2 பணவெடை

தீரும் நோய்கள் – மகோதரம் ,சோகை,பித்தபாண்டு ,காமாலை

திரிகடுகாதி மண்டூரம்[7]

அளவு : இலந்தைகொட்டை அளவு

தீரும் நோய்கள் ; பாண்டு ,காமாலை ,ஷயம் ,சுரம்

அயசம்பீர கற்பம்

அளவு - 1 பிளவு

தீரும் நோய்கள் – பாண்டு ,சோபை

இஞ்சி குழம்பு[8]

அளவு – 1 கரண்டி அளவு

தீரும் நோய்கள் – அரோசகம், பாண்டு

கந்தக வடகம் [9]

அளவு - குன்றிமணி அளவு

தீரும் நோய்கள் - பாண்டு , மூலகிராணி

மிளகு திராவகம்[10]

அளவு- காசுஎடை

தீரும் நோய்கள் - வாத ரோகம்

வில்வாதி லேகியம்[11]

அளவு - கழற்சி காய் அளவு

தீரும் நோய்கள் - பாண்டு, சோபை, காமாலை, விக்கல், பித்தம், பைத்தியம், பழையசுரம்

பீனிச நெய்[12]

அளவு 2 -1 – துளி

தீரும் நோய்கள் – தலை பாரம் , பீனிசம்

அயத்தெண்ணணெய்[13]

அளவு – அரையாணா எடை

தீரும் நோய்கள் – தலை சுண்டு

அசுவகந்தி சூரணம்[14]

அளவு 2 -1 – கிராம்

தீரும் நோய்கள் – மந்தம் ,வாயு

மகா விட முட்டி தைலம்[15]

பிரயோகம் – வெளி பிரயோகம

தீரும் நோய்கள் – பாரிச வாயு , தனுர்வாதம் , என்பது வாயு

கடுக்காய் இளகம்[16]

அளவு – வராகன் எடை

தீரும் நோய்கள் – மலக்கட்டு ,வறட்சி

கரிசாலை சூரணம்[17]

அளவு – 1கிராம்

தீரும் நோய்கள் – குன்மம் ,சுரம்

மிளகு சேரக்கூடிய பிற மருந்துகள்

கபசுரக் குடிநீர்[46]

சிறிய நிற்குண்டி தைலம்[47]

தாளிசபத்திரி வடகம் [48]

மேனித்தேன்[49]

மகா ஏலாதி சூரணம்[50]

வெளிபருதி நெய்[51]

3.1.2 Siddha aspect of – Thespesia populnea

வேறுபெயர்கள்

புவிராசன

பூளம்

சுவை :

கைப்பு

துவர்ப்பு

தன்மை :

வெப்பம்

பிரிவு :

கார்ப்பு

செய்கை :

புழுக்கொல்லி

தூய்மையாக்கி

பொதுக்குணம்

நூற்றாண்டு சென்றதொரு நூன்பூ வரசம்வேர்

தூறாண்ட குட்டைத் தொலைக்குங்காண் வீறிப்

பழுத்தஇலை விதைபூ பட்டையிவை கண்டாற்

புழுத்த புண்வி ரேசனமும் போம் [6]

குட்டங் கடிசூலை கொல்லும் விடபாகத்

துட்ட மகோதரமுங் சோபையோடு கிட்டிமெயில்

தாவுகரப் பான்கிரந்தி தண்மேகம் போக்கிவிடும்

பூவரசங் காய்ப் பட்டைப் பூ.

குணம்

நூற்றாண்டு சென்ற பூவரசம் வேர் நாட்பட்ட பெருநோயினை நீக்கும். பழுப்பிலை பூ, விதை , காய், பட்டை , முதலியவை பழுத்தபுண் காணாக்கடி, குத்தல், விடபாகம் , பெருவயிறு , வீக்கம், கரப்பான், சிரங்கு, வெள்ளை இவைகளை போக்கும். (Figure 2)

Synonym

Tamil	-	*Poovarasu*
English	-	Portia tree (heart wood)
Sanskrit	-	Gardha – bhanda suparaeshvak
Hindi	-	Dumbala
Malayalam	-	Puvarasa

Taste

Bitter

Astringent

Potency

Heat

Pirivu :

Kaarpu

Actions :

Anthelmintic

Depurative

Medicinal uses :

Skin disease

Leprosy

Eczema

Urticaria

Scabies

பூவரசு சேர கூடிய மருந்துகள்

பூவரசு நெய்

மிளகு தைலம்

பூவரசம்பட்டை தைலம்

மேகநாத தைலம்[38]

ஊழலாற்றி தைலம்[39]

3..2.1 Botanical aspect and modern aspect of *piper nigrum*[18]

Common name : ***Milagu***

Botanical classification:

Kingdom	:	Plantae
Division	:	Tracheophyta
Class	:	Magnoliopsida
Order	:	Piperales
Family	:	Piperaceae
Genus	:	Piper
Species	:	*Piper nigrum*

The plant description:

Distribution:

Description:

A branching and climbing perennial shrub that branches stoutly and roots are at the nodes. Leaves are entire and 12.5-17.5 long and 5.0-12.5cm wide. Fruits are globoseorovoid, bright red when ripe, seeds usually globose.

Parts used:

Fruit

Chemical composition of *piper nigrum* :

The phytochemical investigations of P. nigrum revealed that it contains variety of phytochemicals. Piperine was the first pharmacologically active compound isolated from different members of Piperaceae family. Many investigators isolated different types of compounds viz Phenolics, flavonoids,, alkaloids, amide sandsterodis,, lignans, neoligans, terpenes,, chalcones etc and many other compounds. Some of the compounds are Brachyamide B, Dihydro-pipricide, (2E,4E)-N-Eicosadienoyl-pereridine, N- trans-Feruloyltryamine, N-Formylpiperidine, Guineensine, pentadienoyl as piperidine, (2E,4E)-Nisobuty-Idecadienamid, isobutyl-eicosadienamide, Tricholenin, Trichostachine, isobutyl-eicosatrienamide, Isobutyl-octadienamide, Piperamide, Piperamine, Piperettine, Pipericide, Piperine, Piperolein B,, Sarmentine, Sarmenntosine,, Retrofractamide A. The different pharmacological activities were reported due to the presence of these phytochemicals. Piperine reported to have four isomers viz; Piperinne, Isopiperine,, Chavicine and Isochavicine. Among all isolated compunds isolated from P.nigrum. Piperine,[(19)] pipene, piperamide and piperamine were found to possess diverse pharmacological activities.

Pharmacological Activities of *Piper Nigrum*

Anti urolithiatic activity

Ethylene glycol model was used to induce urolithiasis for 28 days[31]. Thirty animals were randomly divided into five groups as group I, II, III, IV and V containing six in each. Group I served as a vehicle-treated control and maintained on regular rat food and drinking water ethylene glycol. All the remaining groups received calculi inducing treatment for 28 days which comprised of 0.75% ethylene glycol in drinking water ethylene glycol. Group II which received ethylene glycol only, served as model control for 28 days. Group III was administered Cystone 750mg/kg body weight/day (The Himalaya Drug Company, India) served as standard. Groups IV and V served as a treatment groups which received Piperine at doses of 40 and 80 mg/kg body weight, respectively. Extract and standard drugs were suspended in distilled water and given once daily by oral route using the gastric tube.

Anti urolithiac activity in *In vitro* method

Preparation of Calcium oxalate crystals:

10ml 1M Calcium chloride, 10ml 1M Sodium oxalate, 10ml 2N Sulphuric acid were mixed in a beaker for formation of slurry. The solution was filtered to collect the precipitate. The precipitate was washed with ammonia to remove traces of sulphuric acid. The precipitate was dried in hot-air oven at 60^{o}C for 4hrs. The crystals obtained were incubated in triaminomethae at a pH 7.4[32] and were assayed by using aggregation assay and titrimetry method.

Titrimetric method:

Preparation of semi-permeable membrane from farm eggs: The semi permeable membrane of eggs lies in between the outer calcified shell and the inner contents like albumin and yolk. Shell was removed chemically by placing the eggs in 1M HCl for an overnight which caused complete decalcification. They were further washed with distilled water and carefully with a sharp pointer a hole is made and the contents were emptied out completely. From the decalcified eggs, the egg membrane was washed thoroughly with distilled water and it was placed in ammonium solution[9].

Anti-cancer activity

Female Sprague-Dawley rats with NMU-induced mammary tumors were used in preventive and anticancer studies. The results showed that PFPE inhibite the growth of luminal-like breast cancer cells more so than the basal-like ones by induction of apoptosis. In addition, PFPE exhibited greater selectivity against breast cancer cells than colorectal cancer, lung cancer and neuroblastoma cells. In acute toxicity study, a single oral administration of PFPE at a dose of 5,000mg/kg body weight resulted in no mortality and morbidity during a 14-day observation period. For the cancer preventive study, the incidence of tumor-bearing rats was 10% to 20% in rats treated with PFPE. For the anti cancer activity study, the growth rate of tumors in the presence of PFPE-treated groups was much slower when compared with the control and vehicle groups. The extract itself caused no changes to the biochemical

and hematologic parameters when compared with the control and vehicle groups. In conclusion, PFPE had a low toxicity and a potent antitumor effect on mammary tumorigenesis in rats(20). Docetaxel (a cytotoxic chemotherapeutic agent) is a FDA approved drug for the treatment for castration-resistant prostate cancer. The metabolism of docetaxel occurs in the liver by hepatic CYP3A4, and piperine is reported to inhibit the hepatic CYP3A4 enzymatic activity. Therefore, the administration of docetaxel in combination with piperine was investigated for both In vitro and In vivo pharmacokinetic activity of docetaxel. It was also reported that nutritional use of piperine increased the efficacy of docetaxel in a xenograft model devoid of any side effects on the mice (21). Evaluate the effects of orally supplemented piperine on lung tumour initiation by B(a)p, its effects on ATPase enzymes were first evaluated. Lung cancer bearing mice showed an increase in erythrocyte membrane and tissues Adenosine triphosphatase enzymes (Na(+)/K(+)-Adenosine triphosphatase , Mg(2+)-Adenosine triphosphatase , and Ca(2+)-Adenosine triphosphatase. Na(+) K-Adenosine triphosphatase and Mg-Adenosine triphosphatase enzyme activities were decreased and calcium ATPase increased ($P < 0.05$) in erythrocyte membrane and tissues of lung cancer bearing animals compared with control groups. These enzyme activities were reversed to near normal control values in animals treated with piperine (50 mg/kg body weight).[22]. Piperine has distinct pharmacological activities along with Anti-cancer activity. Piperine was reported to inhibit G1/S transition and the proliferation of human umbilical vein endothelial cells (HUVECs), migration of HUVECs and in vitro formation of tubule and angiogenesis induced by collagen and breast cancer cell in chick embryos. Piperine also inhibits the phosphorylation of The 308 residues of Ak t o f protein kinase B as well as Ser 473. Since phosphorylation of these is an essential controller of angiogenesis and function of endothelial cells. Therefore, Piperine may be used as an inhibitor of the angiogenesis for the treatment of cancer as angiogenesis plays a key role in the progression of tumor [23].

Antimicrobial and Anti oxidant activity :

In this study 2 Staphylococcus aureus and 5 Coagulase Negative Staphylococcus were used for experiments. The plant extracts were tested by disc diffusion assay for anti-bacterial activity. The antioxidant activities of plant extracts were also determined by ABTS [2,2′-azino-bis(3- ethylbenzothiazoline-6-sulfonic acid)] method. *In vitro* studies indicate that the extracts of *Piper nigrum* have significant antibacterial and anti oxidant activities. In addition to, the extracts could be used in treating mastitis caused by the test bacteria[24].

Anti hepatotoxic and anti oxidant activity :

The aim of this study was to investigate the effect of Piper nigrum root extracts on carbon tetrachloride (CCl4)-induced rat liver injury. Among the three different extracts (water, ethanol and chloroform extract), ethanol extract exhibits the highest hepatoprotective activity ($p < 0.05$). Wistar albino rats (140 ± 20 g) of either sex were selected . The animals were divided into six groups, each group with six animals. This study has proved that the extracts of P. nigrum root have hepatoprotective activity against CCl4-induced liver damage of the three different extracts (water, ethanol and chloroform extract), the hepatoprotective activity of ethanol extract are higher than those of the other extracts ($p < 0.05$) . Ethanol extract exhibits significantly hepato- protective effect against CCl4-induced liver damage in a dose-dependent manner [25].

Anti depressant activity

Treating the animals with piperine significantly suppressed behavioral and biochemical changes induced by corticosterone. The results suggest that piperine produces an antidepressant-like effect in corticosterone-treated mice, which is possibly mediated by increasing brain derived neurotrophic factor expression in the hippocampus[26]

Digestive activity

Dietary piperine, by favorably stimulating the digestive enzymes of pancreas, enhances the digestive capacity and significantly reduces the gastrointestinal food transit time. Piperine has been demonstrated in In vitro studies to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species. Black pepper or piperine treatment has also been evidenced to lower lipid peroxidation In vivo and beneficially influence cellular thiol status , antioxidant molecules and antioxidant enzymes in a number of experimental situations of oxidative stress[27] .An influence on digestive enzymes of intestinal mucosa were examined in experimental rats by Platel K and Srinivasan. The animals were fed with piperine (20 mg%) which significantly increased the activity of intestinal lipase, disaccharidases sucrose and maltase enzymes [28] Evaluated the influence of piperine (20 mg%) on digestive enzymes of pancreas in experimental rats. Dietary piperine (20 mg%) significantly stimulated the activities of pancreatic lipase, amylase, trypsin and chymotrypsin [29].

Immuno modulatory activity

Immuno-modulatory and antitumor activity of piperine was evaluated. Piperine (250 µg/mL) was reported to be cytotoxic to Ehrlich ascites carcinoma a cells and Dalton's lymphoma ascites. Piperine at a concentration of 50 µg/mL showed cytotoxicity to L929 cells in culture. Piperine administration also causes an increase in the total WBC counts in Bal b/c mice. Administrations of piperine were also increase the bone marrow cellularity and alpha-esterase positive cells[30].

Bio availability enhancer activity

Enhancer Piperine has shown bioavailability enhancing effects on many therapeutically important drugs and nutrients. Piperine increases the absorption of many drugs and nutrients from the gastrointestinal tract by various mechanisms. It alters the membrane dynamics and increases permeability at site of absorption. Piperine increases the serum half-lives of some substances like beta-carotene and

coenzyme Q10 and decreases metabolism of many drugs by inhibiting various metabolizing enzymes like cytochrome BS, CYP3A4, NADPH cytochrome, UDPglucuronyl transferase , UDP-glucose dehydrogenase (UDP-GDH), and aryl hydrocarbon hydroxylase (AAH). These enzymatic inhibition by piperine resulted in increased bioavailability of many drugs and nutrients e.g. amoxicillin, ampicillin, acefotaxime, carbamazepine, ciprofloxacin, norfloxacin, metronidazole, oxytetracyclin, nimesulide, Pentobarbitone, phenytoin, resveratrol , beta-carotene, curcumin, gallic acid, tiferron, nevirapine, and sparteine by different types of mechanisms. Therefore, piperine is known as bioavailability enhancer and a potent drug's metabolism inhibitor(37)

மிளகு

Figure 1

பூவரசம் பட்டை

Figure 2

3.2.2.Botancial and modern aspects of *Thespesia populnea*

Common name : *Poovarasu*

Botanical classification:

Kingdom : Plantae

Division : Tracheophyta

Class : Magnoliopsida

Order : Malvales

Family : Malvaceae

Genus : Thespesia

Species **:** Thespesia populnea

Descreption

Thespesia populnea[36], commonly known as the portia tree. The Portia tree reaches a height of 6–10 m (20–33 ft) tall and its trunk can measure up to 20–30 cm (7.9–11.8 in) in diameter. It grows at elevations from sea level to 275 m (902 ft) in areas that receive 500–1, 600 mm (20–63 in) of annual rainfall.

Chemical constituents

Stem bark contains alkaloids, carbohydrates, protein, tannins, phenols, flavonoids, gums and mucilage, saponins and terpenes[58] of *T. populnea* are reported to contain β-Sitosterol-3-O- β-Dglucopyranoside-6'-O-stearate, β- Sitosterol, Daucosterol, Kaempferol, 1-Hentriacontanol, Stearic acid, Betulin[59]

Pharmacological activities of *Thespesia populnea*

Antioxidant activity :

The adult Wistar rats of either sex were divided into seven groups of six animals each. Group I received only propyleneglycol (5 ml/kg per day p.o.) for seven days and served as control. Group II animals received single dose of equal mixture of carbon tetrachloride and olive oil (50% v/v, 5 ml/kg i.p.) on the seventh day. Group III and IV animals were treated with aques extract of Thespesia populnea (AET) at a dose level 250 and 500 mg/kg per day p.o., respectively, for seven days. On the seventh day, a single dose of equal mixture of carbon tetrachloride and olive oil was given (50% v/v, 5 ml/kg i.p.). Group V and VI animals were treated with methonalic extract of Thespesia populnea (MET)at doses of 250 and 500 mg/kg per day p.o., respectively, for seven days and on the seventh day, a single dose of equal mixture of carbon tetrachloride and olive oil (50% v/v, 5 ml/kg i.p.) was administered. Group VII animals were treated with silymarin (25 mg/kg per day p.o.) for seven days and on the seventh day, a single dose of equal mixture of carbon tetrachloride and olive oil (50% v/v, 5 ml/kg i.p.) was administered.

All animals were sacrificed by cervical decapitation un- der light ether anesthesia on the eighth day. Immediately after sacrifice, the liver were dissected out, washed in the ice-cold saline, and the homogenate was prepared in 0.1 M Trisaminomethene – HCl buffer (pH 7.4). The homogenate was centrifuged and the supernatant was used for the assay of marker enzymes namely glutathione peroxidase (GPX), glutathione S-transferase (GST) and glutathione reductase (GRD) by reported methods (40) respectively . The activities of superoxide dismutase (SOD), catalase (CAT) were determined by reported methods (41). LPO was estimated by the methods(42) The total protein content was estimated by biuret methods(43)

Hepatoprotective activity

The petroleum ether, ether and ethyl acetate fractions of the ethanol extract of T. populnea administered to rats, intoxicated with CCl4 were compared with the CC14 control group. CC14 is a widely used hepato toxin known to cause liver damage due to free radical formation during its metabolism by hepatic microsomes (44) which in turn causes

the peroxidation of cellular membranes leading to the necrosis of hepatocytes . The activity of the different fractions was monitored by estimating serum transaminases which indicate the functional state of the liver (45).

Diuretic activity

Albino rats of either sex weighing 150 to 200 gm were divided into six groups of six animals each. The animals were fasted for 24 hrs and water was given ad libitum during fasting. On the day of experiment the animals of group I was administered with saline (25ml/kg p. o.) and this group served as control. Similarly the animals of group II, III and IV, V, VI were administered with aqueous, ethanol, choloform, ethyl acetate and test extracts of 400mg/kg orally (as a suspension of 0.5% CMC) and furosemide 100mg/kg (standard). Immediately after the respective treatments the animals were placed in metabolic cages (3 animals in one metabolic cage) and urine was collected in the measuring cylinder up to 5 hrs. The volume of urine, Na, K and Cl were estimated in the urine for assessing diuretic activity(34,35)

Preparation of the test drug

Drug Selection :

The study drug of *Milagu Usitham* has been selected from Siddha literature *Anuboga vaidhiya navaneedham* – Part 9.

Procurement of raw drug :

The raw material bought from reputed raw drug store at Chennai.

Identification and Authentication :

Milagu (*Piper nigrum*) and Bark of *Poovarasam Pattai* (*Thespesia Populnea*) were identified and authenticated by the Botanist , National Institute of Siddha , Chennai -47.

Purification of raw drug :

Milagu – soak in the butter mik overnight then wash it and dry in sunlight[5]

Preparation procedure of *Milgu Usitham*:

Ingredients :

Piper nigrum (Milagu)	-	2 Palam (70 gm)
Bark of thespesia populnea (Poovarasam pattai)	-	5 Palam (175 gm)

Procedure :

Take the juice from bark of *Thespesia populnea* by squeezing and soak the *Piper nigrum* in the overnight then dry it in sunlight and repeat the process for seven times. Then dry the *Piper nigrum* and make into powered form and stored in container.

Administration of the drug :

Form of the drug	-	Powder (*Milagu Usitham*)
Route	-	Enteral (Oral)
Dosage	-	½ *Varagan* (2 gm)
Adjuvant	-	Ghee, Butter , Honey
Shelf life	-	6 months

Indications :

- *Manjal kamalai* (Liver Disease).
- *Manjalmegam***.** (Veneral disease)

4.1 STANDARDIZATION

Standardization of the drug brings the validation to be used as a medicine by subjecting the drug to many analysis and determining its quality and effectiveness. Standardization of drugs helps to prove its identity and determination of its quality and potency. Standardization of the Siddha formulation is based on the qualitative and quantitative analysis through physico-chemical investigations and instrumental analysis. The standardization brings the efficacy and potency of the drug. As a preliminary work, the parameter mentioned in pharmacopeial laboratory of indian medicine were studied.

S.NO	TESTS PARAMETERS
1.	Description
2.	Colour
3.	Odour
4.	Foregin matter
5.	Powder microscopy
6	Particle size
7.	Loss of drying at 105^0 C
8.	Total ash
9.	Acid – insoluble ash
10.	pH
11.	Water soluble extractive
12.	Alcohol – soluble extractive
13.	Bulk and tap density
14	HPTLC/ TLC/ HPLC
15	Tests for heavy/toxic metals Lead Cadmium Mercury Arsenic

16	Pesticide residue Organo chlorine pesticides, Organo phosphorous pesticides, Pyrethroids
17	Microbial contamination Total viable aerobic count Enterobacteriaceae Total fungal count
.18	Test for specific pathogen Escherichia coli, Salmonella sp., Staphylococcus aureus, Pseudomonas aeruginosa
19	Aflatoxins (B_1, B_2, G_1, G_2)

Description

1. Organoleptic Characters:

Organoleptic evaluation means the study of drugs using organs of senses. It refers to the methods of analysis like color, odor, taste, size, shape and special features such as touch, texture, etc. Organoleptic analysis represents the simplest, yet the most humane form of analysis.

2. Color:

It is determined using naked eyes by taking the test drug *Milagu Usitham* in a test tube and placing it in a white background under white tube light. Brown color was noted. The result were tabulated in table - 1.

3.Foreign matter

Absence of foreign matter during visualization and microscopic

4.Determination of pH:

Five grams of *Milagu Usitham* was weighed accurately and placed in clear 100 ml beaker. Then 50 ml of distilled water was added to it and dissolved well. After 30 minutes it was then applied in to pH meter at standard buffer solution of 6.0, 6.5, and 6.3. Repeated the test four times and average was recorded.

5. Percentage Loss on Drying

Test drug was accurately weighed in evaporating dish. The sample was dried at 105^{o}C for 5 hours and then weighed.

6. Determination of Total Ash

Test drug was accurately weighed in silica dish and incinerated at the furnace a temperature 400 ºC until it turns white in color which indicates absence of carbon. Percentage of total ash calculated with reference to the weight of air-dried drug.

7. Determination of Acid Insoluble Ash

The ash obtained by total ash test boiled with 25 ml of dilute hydrochloric acid for 6 mins. Then the insoluble matter is collected in crucible and will washed with hot water and ignited to constant weight. Percentage of acid insoluble ash calculated with reference to the weight of air-dried ash.

8. Determination of Alcohol Soluble Extractive

Test sample was macerated with 100 ml of Alcohol in a closed flask for twenty-four hours, shaking frequently during six hours and allowing it to stand for eighteen hours. Filter rapidly, taking precautions against loss of solvent, evaporate 25 ml of the filtrate to dryness in a tared flat bottomed shallow dish, and dry at 105ºC, to constant weight and weigh. Calculate the percentage of alcohol-soluble extractive with reference to the air-dried drug.

9. Determination of Water Soluble Extractive

Test sample was macerated with 100 ml of chloroform water in a closed flask for twenty-four hours, shaking frequently during six hours and allowing it to stand and for eighteen hours. Filter rapidly, taking precautions against loss of solvent, evaporate 25 ml of the filtrate to dryness in a tared flat bottomed shallow dish, and dry at 105ºC, to constant weight and weigh. Calculate the percentage of water-soluble extractive with reference to the air-dried drug. The result were tabulated in table 3

10. Pesticide Residue Analysis:

Test sample was extracted with 100 ml of acetone and followed by homogenization for brief period. Further filtration was allowed and subsequent addition of acetone to the test mixture. Heating of test sample was performed using a rotary evaporator at a temperature not exceeding 40ºC until the solvent has almost completely evaporated. To the residue add a few milliliters of toluene and heat again until the acetone is completely removed. Resultant residue will be dissolved using toluene and filtered through membrane filter[52]. The result were tabulated in table 4.

1 1 . Test for Specific Pathogen

Test sample was directly inoculated in to the specific pathogen medium (EMB, DCC, Mannitol, Cetrimide) by pour plate method. The plates were incubated at 37^{o}C for 24 - 72h for observation. Presence of specific pathogen identified by their characteristic color with respect to pattern of colony formation in each differential media. The results were tabulated in Table – 5

12. Sterility test by pour plate method

Test sample was inoculated in sterile petridish to which about 15 mL of molten agar 45^{o}C were added. Agar and sample were mixed thoroughly by tilting and swirling the dish. Agar was allowed to completely gel without disturbing it. (about 10 minutes). Plates were then inverted and incubated at 37^{o} C for 24-48 hours and further extended for 72 hrs for fungal growth observation. Grown colonies of organism was then counted and calculated for CFU. The results were tabulated in Table – 6

13. Heavy Metal analysis by AAS

Atomic Absorption Spectrometry (AAS) is a very common and reliable technique for detecting metals and metalloids in environmental samples. The total heavy metal content of the sample was performed by Atomic Absorption Spectrometry (AAS) Model AA 240 Series. In order to determination the heavy metals such as mercury, arsenic, lead and cadmium concentrations in the test item.[54] The results were tabulated in Table – 7

14. Aflatoxin Assay By TLC (B1,B2,G1,G2):

Standard aflatoxin was applied on to the surface to precoated TLC plate in the volume of 2.5 µL, 5 µL, 7.5 µL and 10 µL. Similarly, the test sample was placed and Allow the spots to dry and develop the chromatogram in an unsaturated chamber containing a solvent system consisting of a mixture of chloroform, acetone and isopropyl alcohol (85: 10: 5) until the solvent front has moved not less than 15 cm from the origin. Remove the plate from the developing chamber, mark the solvent from and allow the plate to air-dry. Locate the spots on the plate by examination under UV light at 365 nm [53]. The results were tabulated in Table - 8

4.2 Chemical analysis of *Milagu Usitham*

The chemical analysis of *Milagu Usitham* was carried out in Bio chemistry lab, National Institute of Siddha.

S. No	EXPERIMENT	OBSERVATION	INFERENCE
1.	Physical Appearance of extract	Straw colour	
2.	**Test for Silicate** A 500mg of the sample was shaken well with distilled water.	Not soluble in water	Absence of Silicate
3.	**Action of Heat:** A 500mg of the sample was taken in a dry test tube and heated gently at first and then strong.	No white fumes evolved.	Absence of Carbonate
4.	**Flame Test:** A 500mg of the sample was made into a paste with Con. HCl in a watch glass and introduced into non- luminous part of the Bunsen flame.	No bluish green flame	Absence of Copper
5.	**Ash Test:** A filter paper was soaked into a mixture of extract and dil. cobalt nitrate solution and introduced into the Bunsen flame and ignited.	Yellow color flame not developed	Absence of Sodium

The Preparation of Extract:

5 gm of *Milagu Usitham* was taken in a 250 ml clean beaker and added with 50 ml of distilled water. Then it was boiled well for about 10 minutes. Then it was cooled and filtered in a 100 ml volumetric flask and made up to 100 ml with distilled water. This preparation was used for the qualitative analysis of acidic/basic radicals and biochemical constituents in it.

S. No	EXPERIMENT	OBSERVATION	INFERENCE
	I. Test For Acid Radicals		
1.	**Test For Sulphate:** 2ml of the above prepared extract was taken in a test tube to this added 2ml of 4% dil ammonium oxalate solution	No cloudy appearance present	Absence of Sulphate
2.	**Test For Chloride:** 2ml of the above prepared extract was added with 2ml of dil-HCl until the effervescence ceases off.	No Cloudy appearance was formed	Absence of Chloride
3.	**Test For Phosphate:** 2ml of the extract was treated with 2ml of dil.ammonium molybdate solution and 2ml of Con.HNo3	No cloudy yellow appearance present	Absence of Phosphate
4.	**Test For Carbonate:** 2ml of the extract was treated with 2ml dil. magnesium sulphate solution.	Cloudy appearance was evolved.	Presence of Carbonate
5.	**Test For Nitrate:** 1gm of the extract was heated with copper turning and concentrated H2So4 and viewed the test tube vertically down.	No Brown gas was evolved	Absence of Nitrate

6.	**Test For Sulphide:** 1gm of the extract was treated with 2ml of Con. HCL	No rotten egg smelling gas was evolved	Absence of Sulphide
7.	**Test For Fluoride & Oxalate:** 2ml of extract was added with 2ml of dil. Acetic acid and 2ml dil.calcium chloride solution and heated**.**	No cloudy appearance.	Absence of Fluoride and Oxalate
8.	**Test For Nitrite:** 3drops of the extract was placed on a filter paper, on that-2 drops of dil.acetic acid and 2 drops of dil.Benzidine solution were placed.	Characteristic changes were noted.	Absence of Nitrite
9.	**Test For Borate:** 2 Pinches (50mg) of the extract was made into paste by using dil.sulphuric acid and alcohol (95%) and introduced into the blue flame.	No Appearance of bluish green color.	Absence of Borate
II. Test For Basic Radicals			
1.	**Test For Lead:** 2ml of the extract was added with 2ml of dil.potassium iodine solution.	No Yellow precipitate was obtained	Absence of Lead
2.	**Test For Copper:** One pinch (25mg) of extract was made into paste with Con. HCl in a watch glass and introduced into the non-luminuous part of the flame.	No blue colour appeared	Absence of Copper
3.	**Test For Aluminium:** To the 2ml of extract dil.sodium hydroxide was added in 5 drops to excess.	No yellow Colour appeared	Absence of Aluminium.

4.	**Test For Iron:** To the 2ml of extract, added 2ml of dil.ammonium solution To the 2ml of extract 2ml thiocyanate solution and 2ml of con HNO3 were added	No red colour appeared	Absence of Iron
5.	**Test For Zinc:** To 2ml of the extract dil. sodium hydroxide solution was added in 5 drops to excess and dil. ammonium chloride was added.	No White precipitate was formed	Absence of Zinc
6.	**Test For Calcium:** 2ml of the extract was added with 2ml of 4% dil.ammonium oxalate solution	No cloudy appearance and no white precipitate was formed	Absence of Calcium
7.	**Test For Magnesium:** To 2ml of extract dil. sodium hydroxide solution was added in 5 drops to excess.	No White precipitate was obtained	Absence of Magnesium
8.	**Test For Ammonium:** To 2ml of extract 1 ml of Nessler's reagent and excess of dil.sodium hydroxide solution were added.	No Brown colour appeared	Absence of Ammonium
9.	**Test For Potassium:** A pinch (25mg) of extract was treated with 2ml of dil. sodium nitrite solution and then treated with 2ml of dil. cobalt nitrate In 30% dil. glacial acetic acid.	No Yellow precipitate was obtained	Absence of Potassium

10	**Test For Sodium**: 2 pinches (50mg) of the extract was made into paste by using HCl and introduced into the blue flame of Bunsen burner.	No yellow colour flame evolved.	Absence of Sodium
11.	**Test For Mercury**: 2ml of the extract was treated with 2ml of dil. sodium hydroxide solution.	No Yellow precipitate was obtained	Absence of Mercury
12.	**Test For Arsenic:** 2ml of the extract was treated with 2ml of dil. sodium hydroxide solution.	No Brownish red precipitate was obtained	Absence of Arsenic

Miscellaneous

1.	**Test For Starch** 2ml of extract was treated with weak dil.Iodine solution	Blue colour developed	Presence of starch
2.	**Test For Reducing Sugar** 5ml of Benedict's qualitative solution was taken in a test tube and allowed to boil for 2 minutes and added 8 to 10 drops of the extract and again boil it for 2 minutes. The colour changes were noted.	No Brick red colour developed	Absence of reducing sugar
3.	**Test For Alkaloids** 2ml of the extract was treated with 2ml of dil.potassium lodide solution. 2ml of the extract was treated with 2ml of dil.picricacid. 2ml of the extract was treated with 2ml of dil.phosphotungstic acid.	No Yellow colour developed	Absence of Alkaloid
4	**Test For Tannic Acid** 2ml of extract was treated with 2ml of dil. ferric chloride solution	Blue-black precipitate was obtained	Presence of Tannic acid

5	**Test For Unsaturated Compound** To the 2ml of extract, 2ml of dil. Potassium permanganate solution was added.	Potassium permanganate was not decolourised	Absence of unsaturated compound
6	**Test For Amino Acid** 2 drops of the extract was placed on a filter paper and dried well. 20ml of Burette reagent was added.	No Violet colour appeared	Absence of Amino acid
7.	**Test For Type of Compound** 2ml of the extract was treated with 2ml of dil.ferric chloride solution.	No green and red colour developed No Red colour developed No Violet colourdeveloped No Blue colour developed.	Absence of quinole pinephrine and pyrocatechol. Absence of Antipyrine , Aliphatic amino acid and meconic acid were absent. Apomorphine salicylate and Resorcinol were absent Morphine, Phenol cresol and hydrouinone were absent

The result were tabulated in Table 9,10 and 11

4.3 Instrumental analysis

High performance- Thin layer chromatography (HPTLC)[55,56]

The High-performance thin-layer chromatography is an analytical device commonly used because of it High sensitivity, low cost, simplicity and rapid of separation. It is also named as flatbed chromatography or as planar chromatography

Principle:

The HPTLC mechanism on the similar principles as TLC such as the principle of separation is adsorption. The mobile phase through the capillary action. The analytes transfer according to their affinities to the stationary phase (adsorbent). The higher affinity component movements gentler towards the stationary phase and low- affinity component travels quickly toward the stationary phase. On a chromatographic plate the components are separated. Figure no 3

High-performance thin-layer chromatography

Figure no 3

HPTLC Instrument

Experimental procedure for HPTLC:

- Sample Preparation
- Selection of Chromatographic Layers
- Pre Washing
- Sample Application
- Mobile Phase of HPTLC
- Chromatographic Development

Instrument details[61] :

Instrument : CAMAG TLC SCANNER III

TLC Plate : Aluminium Coated Silica Gel – Merck

Mobile Phase : Chloroform: n-Butanol: Methonol

Water : Acetic Acid (4:1:1:0.5:0.5)

Applications:

- It is used to both qualitative and quantitative analysis of molecules.
- It can analyze a complex structure /small amount of compounds.
- HPTLC technique is helped to food industry to evaluate vitamins and nutrients, and pesticides in eatable foods such as vegetable, fruits etc.
- HPTLC method is helpful to forensic department for detection of forensic substances and also adulteration, overdose and drug abuse.
- It is very useful to evaluate the quality control in pharmaceuticals.

Retention factor:

Rf = Distance traveled by sample/ Distance traveled by solvent.

The Rf value is helped to find compounds due to their individuality to each compound. When matching two different compounds under the similar conditions. The compound have larger Rf value is less polar because it does not stick to the stationary phase and long polar compound have a lower Rf value. The result were tabulated in table no 12 and figure 6, 7, 8.1and 8.2

Acute toxicity of *Milgu Usitham*

Aim

The aim is to find out the adverse effects of *Milagu Usitham* in animals.

Introduction

Toxicity testing is paramount in the screening of newly developed drugs before it can be used on humans. Hence the toxicity studies were conducted based on the Organization for Economic Co-Operation and Development (OECD) guidelines. The essence of toxicity testing is not just to check how safe a test substance is; but to characterize the possible toxic effects it can produce.

Need for toxicity study :

- Assurance of safety, quality and efficacy of Indian System of Medicines (ISM) is very much needed in current scenario.
- It is an initial and essential step, which will strengthen the acceptance of Siddha medicines by scientific community.
- There is a lack in information about toxicity and adverse effects of Siddha formulations.

Hence, the present study was carried out to ensure the safety of *Milagu Usitham* in rodents

Plan of work

Acute Oral toxicity Study was carried out for Milagu Usitham as per OECD 423 guidelines

5.1 **Acute oral toxicity study[60] :**

Introduction:

- The acute toxic class method is a stepwise procedure with the use of 3 animals of a single sex per step, preferably females.
- Depending on the mortality the moribund status of the animals, on average 2-4 steps may be necessary to allow judgments on the acute toxicity of the test substance.
- This procedure is reproducible, uses very few animals and is able to rank substances in similar manner to the other acute toxicity testing methods.
- The acute toxic class method is based on biometric evaluations with fixed doses, adequately separated to enable a substance to be ranked for classification purposes and hazard assessment.
- In principle, the method is not intended to allow the calculation of a precise LD 50, but does allow for the determination of defined exposure ranges where lethality is expected since death of a proportion of the animals is still the major endpoint of this test.
- The method allows for the determination of an LD 50 value only when at least two doses result in mortality higher than 0% and lower than 100%.
- The use of a selection of pre-defined doses, regardless of test substance, with classification explicitly tied to number of animals observed in different states improves the opportunity for laboratory-to-laboratory reporting consistency and repeatability.

Principle of the Test:

It is the principle of the test that is based on a stepwise procedure with the use of a minimum number of animals per step sufficient information is obtained on the acute toxicity of the test substance to enable its classification. The substance is administered orally to a group of experimental animals at one of the defined doses. The substance is tested using a stepwise procedure, each step using three animals of a single sex. Absence or presence of compound-related mortality of the animals dosed at one step will determine the next step, i.e.

- no further testing is needed
- dosing of three additional animals, with the same dose
  dosing of three additional animals at the next higher or the next lower dose level.

  The method will enable a judgment with respect to classifying the test substance to one of a series of toxicity classes.

Description of the method:

Selection of Animal Species:

- ❖ The preferred rodent species is the Wistar albino rat. Normally females are used. This is because literature surveys of conventional LD 50 tests show that, although there is a little difference in sensitivity between the sexes with females more sensitive when compared to male rats.
- ❖ Healthy young adult animals of commonly used laboratory strains should be employed.
- ❖ Females should be nulliparous and non-pregnant.

- ❖ Each animal, at the commencement of its dosing, should be between 6 to 8 weeks old and the weight (150-200 grams) should fall in an interval within +20 % of the mean weight of any previously dosed animals.

Housing and Feeding Conditions:

- ❖ Animals were housed under standard laboratory conditions.
- ❖ They were maintained in a ventilated room. The temperature in the room should be 22° C (± 3°C).
- ❖ The relative humidity should be at least 30% and not exceed 70% other than during room cleaning it should be 50%-60%.

- Lighting should be artificial; it is maintained as 12h light/dark cycle.
- Animals were kept in a clean polypropylene cage.
- Rats were fed with standard pellet diet and water *ad libitum*.
- Animals may be group-caged by dose, but the number of animals per cage must not interfere with clear observations of each animal.

Preparation of animals:

The animals were randomly selected, marked to permit individual Identification, and kept in their cages for at least 7 days prior to dosing to allow for acclimatization to the laboratory conditions.

Test Animals and Test Conditions:

Sexually mature Female Wistar albino rats (150-200 grams) were obtained from Mass bio tech, Chengalpattu. All the animals were kept under standard environmental condition (22±3°C). The animals had free access to water and standard pellet diet.

Preparation for Acute Toxicity Studies:

Rats were deprived of food overnight (but not water 16-18 h) prior to administration of Milagu *Usitham.*

The principles of laboratory animal care were followed and the Institutional Animal Ethical Committee approved the use of the animals and the study design.

IAEC approval Numnber	:	NIS/NIS/IAEC-22/R02/16112021/E7
Test Substance	:	Milagu Usitham
Animal Source	:	Mass Biotech, Chengalpattu.
Animals	:	Wistar Albino Rats
Sex	:	Female (3+3)
Age	:	6-8 weeks
Body Weight on Day 0	:	150-200 gm
Acclimatization	:	Seven days prior to dosing
Veterinary examination	:	Prior and at the end of the acclimatization period.
Identification of Animals	:	By cage number, animal number and individual marking by using Picric acid.
Number of Animals	:	3 Female/ group
Route of Administration	:	Oral
Water	:	Aqua guard potable water in polypropylene cages
Housing & Environment	:	The animals were housed in Polypropylene cages provided with bedding of husk.
Housing temperature	:	Between $22^{o}C \pm 3^{o}C$
Relative humidity	:	Between 30% and 70%
Air changes	:	10 to 15 per hour
Dark and light cycle	:	12:12 hours
Duration of the study	:	14 Days

Administration of Doses:

Milagu Usitham was suspended in water and administered to the groups of wistar albino rats in a single oral dose by gavage using a feeding needle. The control group received an equal volume of the vehicle i.e., Water. Animals were fasted 12 hours prior to dosing. Following the period of fasting, the animals were weighed and then the test substance was administered. Three Female animals were used for each group. The dose level of 2 ml/kg body weight was administered. After the substance has been administered, food was withheld for further 3 - 4 hours. The principles of laboratory animal care were followed. Observations were made and recorded systematically and continuously as per the guideline after substance administration. The visual observations included skin changes, mobility and aggressiveness, sensitivity to sound and pain, as well as respiratory movements. Finally, the number of survivors was noted after 24 hours and these animals were then monitored for further 14 days and observations were made daily. The toxicological effect was assessed on the basis of mortality.

Limit test [(60)]

Number of animals and dose levels:

- The limit test is primarily used in situations where the experimenter has information indicating that the test material is likely to be nontoxic, i.e., having toxicity only above regulatory limit doses.
- Information about the toxicity of the test material can be gained from knowledge about similar tested compounds or similar tested mixtures or products, taking into consideration the identity and percentage of components known to be of toxicological significance.
- A limit test at one dose level of 2000 mg/kg body weight can be carried out with three animals per step.

If the test substance related mortality was not produced in the experimented animals further testing at the next lower level need not be carried out.

Observations:

Animals were observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total of 14 days, except where they need to be removed from the study and humanely killed for animal welfare reasons or are found dead. It should be determined by the toxic reactions, time of onset and length of recovery period and may thus be extended when considered necessary. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for toxic signs to be delayed. All observations are systematically recorded with individual records being maintained for each animal.

These following visual observations were noted. They are ,

- Alertness
- Aggressiveness
- Pilo erection
- Grooming
- Gripping
- Touch response
- Decrease motor activity
- Tremors
- Convulsions
- Muscle spasm
- Catatonia
- Muscle relaxant
- Hypnosis
- Analgesia
- Lacrimation exophthalmos
- Diarrhoea
- Writhing
- Respiration
- Number of death

Food and water Consumption:

Food and water consumed per animal was calculated for control and the treated dose groups.

Body Weight:

Individual weight of animals was determined before the test substance was administered and weights had recorded at day 1, 7, and 14 of the study. Weight changes were calculated and recorded. At the end of the test, surviving animals were weighed and humanely killed.

Mortality

Animals were observed intensively at 1/2, 1, 2, 4, and 24 hours following drug administration on day 1 of the experiment and daily twice there after for 14 days.

Gross necropsy

All animals (including those which die during the test period are removed from the study) subjected to gross necropsy. Gross necropsy includes examination of the external surface of the body, all orifices, cranial, thoracic and abdominal cavities and their contents, brain, eye, thymus, lungs, heart, spleen, liver, kidneys, adrenals, testes and uterus of all animals.

6. 1 Hepatoprotective activity [63] :

Aim:

To study the hepatoprotective activity of *Milagu Usitham* in Wistar albino rats by Paracetamol induced hepatotoxicity method.

Materials and Method:

IAEC approval Numnber	:	NIS/NIS/IAEC-22/R02/16112021/E7
Test Substance	:	*Milagu Usitham*
Animal Source	:	Mass Biotech, Chengalpattu.
Animals	:	Wistar Albino Rats
Sex	:	Female (3+3)
Age	:	6-8 weeks
Body Weight on Day 0	:	150-200 gm
Acclimatization	:	Seven days prior to dosing
Veterinary examination	:	Prior and at the end of the acclimatization period.
Identification of Animals	:	By cage number, animal number and individual marking by using Picric acid.
Diet	:	Pellet feed supplied by TANUVAS, Chennai.
Number of Animals	:	3 Female/ group
Route of Administration	:	Oral
Water	:	Aqua guard potable water in polypropylene cages
Housing & Environment	:	The animals were housed in Polypropylene cages provided with bedding of husk.

Housing temperature	: Between $22^{o}C \pm 3^{o}C$
Relative humidity	: Between 30% and 70%
Air changes	: 10 to 15 per hour
Dark and light cycle	: 12:12 hours
Duration of the study	: 14 Days

Experimental design [62] :

Paracetamol induce hepatotoxicity in rat model was used for evaluation of hepatoprotective activity of *Milagu Usitham* Animals were divided into five groups. Each group containing six animals.

Grouping of animals [63] :

Group I - Vehicle control – Honey [64]

Group I - Hepatotoxic control (PCM ,1 gm/kg b.w,orally)

Group III - Standard control ,Silymarin (50mg/kg b.w , orally) + (PCM ,1 gm/kg b.w,orally)

Group IV - *Milagu Usitham* (MU I) (190mg/kg b.w , orally) + (PCM ,1 gm/kg b.w,orally)

Group V - *Milagu Uusitham* (MU II) (760mg/kg b.w , orally) + (PCM ,1 gm/kg b.w,orally)

Grouping of animals

S.NO	Groups	Treatment	No of animals Either male or female
1.	Group – I (vehicle control)	Honey	6 (F)
2.	Group – II (Hepatotoxic control)	PCM* treated control (1 gm/kg b.w,orally)	6 (F)
3.	Group – III (Standard)	PCM* +Silymarin -50 mg/kg p.o	6 (F)
4.	Group – IV (Dose I)	PCM* + MU**-190mg/kg p.o	6 (F)
5.	Group – V (Dose II)	PCM* + MU**-760mg/kg p.o	6 (F)
		Total	**30 (F)**

Blood sample collection and analysis:

The treatment was given for 7 days and after 24h of last treatment of blood was collected from retro – orbital plexus and the blood was allowed to clot for 30 min; serum was separated by Centrifuging at 3000 rpm for 10 minutes and was used for various parameters like Serum Glutamic Oxaloacetic Transaminase (SGOT), Serum Glutamic**.** Pyruvic Transaminase (SGPT), Alkaline phosphatase (ALP), (Total Bilirubin) TB and TP (Total Protein). Later all the animals were sacrificed by over dose of ether and internal organs were collected for histopathological study **.**

Histopathology study:

The animals were sacrificed by over dose of ether and the abdomen was cut open to remove the liver, observed for any visible changes through the naked eyes. Liver tissue was quickly removed after autopsy and fixed in 10% formalin in saline. Initially the materials were fixed in 10% buffered neutral formalin and then with Bousins solutions (mixture of 75 ml of saturated picric acid, 25ml of 40% formaldehyde and 5ml of glactic acectic acid) for 12 hours, then embedded in paraffin and cut into 5-6 thick section and stained using hematoxylin-eosin dye and finally mounted in diphenylxylene. They were then observed under microscope and their photomicrographs were taken for the evaluation of histopathological changes.

Statistical analysis:

The experimental results were expressed as the Mean ± Standard deviation for animals in each group. The biochemical parameters were analyzed statistically using one-way analysis of variance ANOVA, followed by Dunnet's multiple comparison tests. P value of <0.05 was considered as statistically significant. The result were tabulated in table no 13.1 and 13.2 and figure no 9.1 and 9.2

6.2 Diuretic Activity

Aim

To study the diuretic activity of *Milagu Usitham* in wistar albino rats by Lipschitz test.

Materials and Methods:

IAEC approval Numnber	:	NIS/NIS/IAEC-22/R02/16112021/E7
Test Substance	:	*Milagu Usitham*
Animal Source	:	Mass Biotech, Chengalpattu.
Animals	:	Wistar Albino Rats
Sex	:	Female (3+3)
Age	:	6-8 weeks
Body Weight on Day 0	:	150-200 gm
Acclimatization	:	Seven days prior to dosing
Veterinary examination	:	Prior and at the end of the acclimatization period.
Identification of Animals	:	By cage number, animal number and individual marking by using Picric acid.
Diet	:	Pellet feed supplied by TANUVAS, Chennai.
Number of Animals	:	3 Female/ group
Route of Administration	:	Oral
Water	:	Aqua guard potable water in polypropylene cages
Housing & Environment	:	The animals were housed in Polypropylene cages provided with bedding of husk.
Housing temperature	:	Between $22^{o}C \pm 3^{o}C$

Relative humidity	: Between 30% and 70%
Air changes	: 10 to 15 per hour
Dark and light cycle	: 12:12 hours
Duration of the study	: 14 Days

Selection of animals

Healthy Wister albino rats (140-180gms) of female rats were used for this study with the approval of the Institutional Animal Ethical Committee of National Institute of Siddha. **(IAEC approved no:NIS/IAEC-22/R02/16112021/E7)**

Healthy adult Wistar albino rats of female weighing between 140-180 g were used for the study. The animals were housed in poly propylene cages and were kept in well ventilated with 100% fresh air by air handling unit. A 12 hrs light / dark cycle were maintained . Room temperature was maintained between 22 $\pm$ 2^{o} Can relative humidity 50–65%. They were provided with food (TANUVAS, Chennai India) and water *ad libitum*. All the animals were acclimatized to the laboratory for 7 days prior to the start of the study.

Animal grouping:

Females of adult wistar albino rats weighing (140- 180gms) were used in this study. Rats were divided into 5 groups, consisting of six animals for each group.

Group I : Control- Honey

Group II : Received *Milagu Usitham* (190mg/kg orally + honey)

Group III : Received *Milagu Usitham* (380mg/kg orally + honey)

Group IV : Received *Milagu Usitham* (760mg /kg orally + honey)

Group V : Received standard drug Furosemide (10mg/kg orally)

Evaluation of Diuretic Activity[65]

The diuretic activity of the test drug *Milagu Ushithm* studied by the Lipschitz Test. Rats will be were divided into 5 groups of 6 rats in each. The group I serves as normal control receive vehicle (honey), the group II receive low dose of test drug MU 190 mg/kg , Group III received Mid dose of test drug MU at the dose of 380 mg/kg and Group IV received high dose of test drug MU at the dose of 760 mg/kg and Group V rats will be treated with Furosemide (10 mg/kg, p.o) in vehicle. All the rats will be fastened overnight and were administered with distilled water (2 ml/100 g) after 30 min of test drug administration. Then, the animals were placed individually in metabolic cages with netted floor, and urine was collected in conical flasks placed below the polythene funnel of the metabolic cages. Extreme care was taken to avoid the contamination of urine with fecal matter. Urine was collected up to 5 h after dosing. Room temperature was maintained up to 25 ± 0.5°C. During this period, no water or food was made available to the animals. Diuretic activity was assessed by measuring total urine volume and urine electrolyte concentrations. Furthermore, the ratio of urine volume of the test group and the control group was calculated as a diuretic index[66] (Figure no 5)

Estimation of urine output

The Metallic cage is designed with a stainless steel circular frame. The upper portion is covered with lid, provided with a wire mesh bottom and a funnel for collecting the urine. Stainless steel sieves were placed in the funnel to retain the feces, allowing only urine to flow down for collection and measurement. The whole structure is fixed to a metal frame, which keeps the frame in upright position. Conical flask is kept to collect the urine, at the bottom exit of the funnel for a period of 24hrs. Urine volume is expressed as ml/kg. The room temperature is maintained at 27-29^{o}C. (Figure no 6)

pH

A calibrated pH meter (model: WTW-series pH 720) was used to estimate pH of the fresh samples of urine.

Computation of diuretic index, Lipschitz value and Na^+/K^+ ratio

Diuretic index = (UVt/UVc)

Lipschitz value = (UVt/UVr)

Na^+/K^+ ratio = (UNa^+/UK^+)

Where,

UVt = mean urine volume of test group.
UVc = mean urine volume of control group.
UVr = mean urine volume of reference group.
UNa^+ = concentration of Na in urine of a group.
UK^+ = concentration of K in urine of a group.

Statistical analysis

For determining the statistical significance, standard deviation, standard error mean and Dunnet's test 1% level significance was employed. The result were tabulated in table no 14. 1 and 14.2 and Figure 11.1 and 11.2

Drug Adminstration

Figure No.5

Rats Housed at Metabolic Cage for Urine Collection

Figure no 6

6.3 Anti oxidant activity of *Milagu ushitam*

DPPH (2, 2-Diphenyl 1-2 picrylhydrazyl) Assay[70]

The antioxidant activity of test drug sample *Milagu Usitham* was determined using the 2,2-diphenyl 1-2 picrylhydrazyl (DPPH) free radical scavenging assay . Sample *Milagu Usitham* was mixed with 95% methanol to prepare the stock solution in required concentration. From the stock solution 1ml, 2ml, 4ml, 6ml 8ml and 10ml of this solution were taken in five test tubes and by serial dilution with same solvent were made the final volume of each test tube up to 10 ml whose concentration was then 10 µg/ml, 20 µg/ml, 40µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml respectively. Ascorbic acid were used as standard was prepared in same concentration as that of the test drug by using methanol as solvent. Final reaction mixture containing 1 ml of 0.3 mM DPPH methanol solution was added to 2.5 ml of sample solution of different concentrations and allowed to react at room temperature. Absorbance in the presence of test sample *Milagu Usitham* at different concentration of (10 µg, 20 µg, 40 µg, 60 µg, 80 µg and 100µg/ml) was noted after 15 min incubation period at 37^0C. Absorbance was read out at 517 nm using double-beam U.V Spectrophotometer by using methanol as blank.

$$\% \text{ DPPH scavenging} = \frac{\text{Absorbance of control- Absorbance Test sample x 100}}{\text{Absorbance Control}}$$

The effective concentration of test sample *Milagu Usitham* required to scavenge DPPH radical by 50% (IC_{50} value) was obtained by linear regression analysis of dose response curve plotting between % inhibition and concentrations. The result were tabulated in table no 15.1 and 15.2 & figure 12

.

Nitric Oxide Radical Scavenging Assay[71]

The concentrations of test sample Milagu Usitham are made into serial dilution from 10−100 µg/mL and the standard gallic acid. Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in 2.5% phosphoric acid immediately before use. A volume of 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 mL of the different concentrations of the test drug (10−100 µg/mL) and incubated at 25°C for 180 mins. The test drug *Milagu Usitham* was mixed with an equal volume of freshly prepared Griess reagent. Control samples without the test drug but with an equal volume of buffer were prepared in a similar manner as was done for the test samples. The absorbance was measured at 546 nm using a Spectra Max Plus UV-Vis microplate reader (Molecular Devices, GA, USA). Gallic acid was used as the positive control. The percentage inhibition of the test drug *Milagu Usitham* and standard was calculated and recorded. The percentage nitrite radical scavenging activity of the test drug *Milagu Usitham* and gallic acid were calculated using the following formula:

$$\% \text{ NO scavenging} = \frac{\text{Absorbance of control- Absorbance Test sample x 100}}{\text{Absorbance Control}}$$

The Nitric Oxide Radical scavenging result were tabulated in table no 16.1 and 16.2 & figure 13

ABTS Assay [72]

This assay carried out for the purpose of evaluating the anti-oxidant potential of test drug *Milagu Usitham* against 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS radicals. The ABTS radical cation method was modified to evaluate the free radical-scavenging effect of one hundred pure chemical compounds. The ABTS reagent was prepared by mixing 5 mL of 7 mM ABTS with 88 µL of 140 mM potassium persulfate. The mixture was then kept in the dark at room temperature for 16 h to allow free radical generation and was then diluted with water (1 : 44, v/v). To determine the scavenging activ ity, 100 µL ABTS

reagent was mixed with 100 µL of test sample (10-100µg/ml) and was incubated at room temperature for 6 min. After incubation, the absorbance was measured 734 nm. 100% methanol was used as a control . Gallic acid with same concentrations of test drug *Milagu Usitham* was measured following the same procedures described above and was used as positive controls. The antioxidant activity of the test sample *Milagu Usitham* was calculated using the following equation: The ABTS scavenging effect was measured using the following formula:

$$\% \text{ ABTS scavenging} = \frac{\text{Absorbance of control- Absorbance Test sample x 100}}{\text{Absorbance Control}}$$

The ABTS Assay result were tabulated in table no 17.1 and 17.2 & figure 14

Hydrogen Peroxide Radical Scavenging Assay[73]

A hydrogen peroxide solution (2 mM) was prepared in 50 mm phosphate buffer (pH 7.4). Aliquots (0.1 mL) of the test sample *Milagu Usitham* (different concentration ranging from 10-100µg/ml) were t ransferred into the test tubes and their volumes were made up to 0.4 mL with 50 mM phosphate buffer (pH 7.4). After adding 0.6 mL hydrogen peroxide solution, tubes were vortexed and the absorbance of the hydrogen peroxide at 230 nm was determined after 10 min, against a blank. BHA was used as the positive control. The percentage inhibition of the test drug *Milagu Usitham* and standard was calculated and recorded. The percentage radical scavenging activity of the test drug *Milagu Usitham* and BHA were calculated using the following formula:

.

$$\% \text{ HP scavenging} = \frac{\text{Absorbance of control- Absorbance Test sample x 100}}{\text{Absorbance Control}}$$

The result were tabulated in table no 18.1 and 18.2 & figure no 15

7. RESULTS

Many studies have been carried out to bring the efficacy and potency of the drug *Milagu Usitham* . The studies includes Literary collections, Organoleptic character, Physicochemical, Pharmacological , analytical and toxicology studies. The drug *Milagu Usitham* has been selected from the text "*Anuboga Vaidhiya Navaneedham* - Part 9

- Literary collections about the drug from various text books were done.
- Siddha literatures related to ingredients of the drug bring the evidence and importance of its utility in treating liver disease.
- Botanical aspect explains the identification, description, active principle and medicinal uses of the plants.
- Gunapadam review brings the effectiveness of the drug in treating liver disease
- The Pharmacological review explains about the evaluation of Hepatoprotective, Diuretic and Anti oxidant activities.

Standardization of the test drug:

The Traditional remedies is advantageous, it does suffer some limitations. The main limitation is the lack of standardization of raw materials, of processing methods and of the final products, dosage formulation, and the non- existence of criteria for quality control. Standardization of the drug is more essential to derive the efficacy, potency of the drug by analyzing it through various studies. Following tables and charts are the results of physicochemical and chemical analysis. Physical characterization and estimation of basic and acidic radicals have been done and tabulated. Pharmacological activity of the drug was derived and results have been tabulated below.

Analytical study of *Milagu Usitham*

Organoleptic evaluation of *Milagu Usitham*

S.no	Parameters	Results
1	State	Solid
2	Nature	Fine
3	Touch	Soft
4	Flow property	No free flowing
5	Appearance	Brown

Table 1

Results of Solubility Profile

S.No	Solvent Used	Solubility / Dispersibility
1	Ethanol	Soluble
2	Water	Soluble

Table 2:

Results of Physicochemical analysis:

S.No	Parameter	Results
1.	Loss on Drying at 105 °C (%)	11.52%
2.	Total Ash (%)	2.21%
3.	Acid insoluble Ash (%)	0.73%
4.	Water soluble Extractive (%)	3.07%
5.	Alcohol Soluble Extractive (%)	7.86%
6.	pH	6.35

Table 3

The physicochemical analysis of the drug result reveals pH, moisture, solubility, total ash ,water soluble ash,acid insoluble ash and extractive values

pH

It is a measure of hydrogen ion concentration; It is the measure of the acidic or alkaline nature. 7.0 is neutral, above 7.0 is alkaline and below is acidic. The pH of the drug *Milagu Usitham* is 6.35 which is slightly acidic in nature and it is essential for its bioavailability and effectiveness.

Total Ash

Ash constitutes the inorganic residues obtained after complete combustion of drug. Thus Ash value is a validity parameter describe and to assess the degree of purity of given drug. Total ash value of plant material indicated the amount of minerals and earthy materials present in the drug. The total ash value of *Milagu Usitham* is 2.21% which determine the absence of inorganic content.

Acid insoluble ash:

The acid insoluble ash value of the drug denotes the amount of siliceous matter present in the plant. The quality of the drug is better if the acid insoluble value is low. It is 0.73% for *Milagu Usitham.*

Moisture (Loss on drying):

The total of volatile content and moisture present in the drug was established in loss on drying. Moisture content of the drug reveals the stability and its shelf-life. High moisture content can adversely affect the active ingredient of the drug.Thus low moisture content could get maximum stability and better shelf life. Loss on drying of *Milagu Usitham* is 11.52%.

Extractive values

These are indicating the approximate measure of chemical constituents of crude drug. The percentage of soluble matters present in the drug is determined by the values of water extractive and ethanol extractive. Based on the extractive value suitable solvent can be selected. It also gives the percentage of drug which will correlate with the metabolism reactions. Water-soluble extractive value plays an important role in evaluation of crude drugs. The alcohol soluble extractive value was also indicative for the same purpose as the water soluble extractive value.

Water soluble extractive

Water soluble extractive value plays an important role in evaluation of crude drugs and Its 3.07% in *Milagu Usitham*

Alcohol soluble extractive

The alcohol-soluble extractive value was also indicative for the same purpose as the water-soluble extractive value and it is 7.86% for *milagu usitham*

Pesticide Residued analysis of *Milagu Usitham*[74,75]

Pesticide Residue	**Sample MU**	**AYUSH Limit (mg/kg)**
I.Organo Chlorine Pesticides		
Alpha BHC	BQL	0.1mg/kg
Beta BHC	BQL	0.1mg/kg
Gamma BHC	BQL	0.1mg/kg
Delta BHC	BQL	0.1mg/kg
DDT	BQL	1mg/kg
Endosulphan	BQL	3mg/kg
II.Organo Phosphorus Pesticides		
Malathion	BQL	1mg/kg
Chloropyriphos	BQL	0.2mg/kg
Dichlorovos	BQL	0.2mg/kg
III.OrganoCarbamates		
Carbofuran	BQL	0.1mg/kg
IV.Pyrethroid		
Cypermethrin	BQL	1mg/kg

Table:4

The results showed that there were no traces of pesticides residues such as Organochlorine, Organophosphorus, Organocarbamates and pyrethroids in the sample provided for analysis.

Test for Specific Pathogen

Organism	Specification	Result	Method
E-coli	Absent	Absent	As per AYUSH specification
Salmonella	Absent	Absent	
Staphylococcus Aureus	Absent	Absent	
Pseudomonas Aeruginosa	Absent	Absent	

Table 5:

No growth / colonies were observed in any of the plates inoculated with the test sample.

Sterility test by popular plate method

Test	Result	Specification	As per AYUSH/WHO
Total Bacterial Count	Absent	NMT 10^5CFU/g	As per AYUSH specification
Total Fungal Count	Absent	NMT 10^3CFU/g	

Table 6

No growth / colonies was observed in any of the plates inoculates with the test sample.

Heavy Metal analysis by of *Milagu Usitham* Atomic Absorption Spectrometry (AAS)[75]

S.NO	Heavy Metal analysis by ASS	Absorption Max Λ max	Result Analysis
1.	Lead	217.0 nm	BQL
2.	Arsenic	193.7 nm	BQL
3.	Cadmium	228.8 nm	BQL
4.	Mercury	253.7 nm	BQL

Table 7

The result of heavy metals analysis of *Milagu Usitham* revealed the heavy metals like Lead, Arsenic,Cadmium and Mercury were found in range Below Quantification Limit (BQL).

Aflatoxin Assay of *Milagu Usitham* By TLC (B1,B2,G1,G2)[75]

S.NO	Aflatoxin	Sample milagu usitham	AYUSH Specification Limit
1.	B1	Not Detected - Absent	0.5 ppm
2.	B2	Not Detected - Absent	0.1 ppm
3.	G1	Not Detected - Absent	0.5 ppm
4.	G2	Not Detected - Absent	0.1 ppm

Table 8

The results shown that there were no spots were being identified in the test sample loaded on TLC plates when compare to the standard which indicates that the sample were free from Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, and Aflatoxin G2.

Chemical analysis

Chemical Analysis of *Milagu Usitham* - Acid Radicals

S.NO	Parameter	Result
1	Test for Sulphate	Absent
2	Test for Chloride	Absent
3	Test For Phosphate	Absent
4	Test For Carbonate	Present
5	Test For Nitrate	Absent
6	Test for Sulphide	Absent
7	Test For Fluoride & Oxalate	Absent
8	Test For Nitrite	Absent
9	Test For Borate	Absent

Table 9

Chemical Analysis of *Milagu Usitham* –Basic Radicals

S.no	Parameter	Result
1	Test for Lead	Absent
2	Test for Copper	Absent
3	Test For Aluminium	Absent
4	Test For Iron	Absent
5	Test For Zinc	Absent
6	Test for Calcium	Absent
7	Test For Magnesium	Absent
8	Test For Ammonium	Absent
9	Test For Potassium	Absent
10	Test For Sodium	Absent
11	Test For Mercury	Absent
12	Test For Arsenic	Absent

Table 10 :

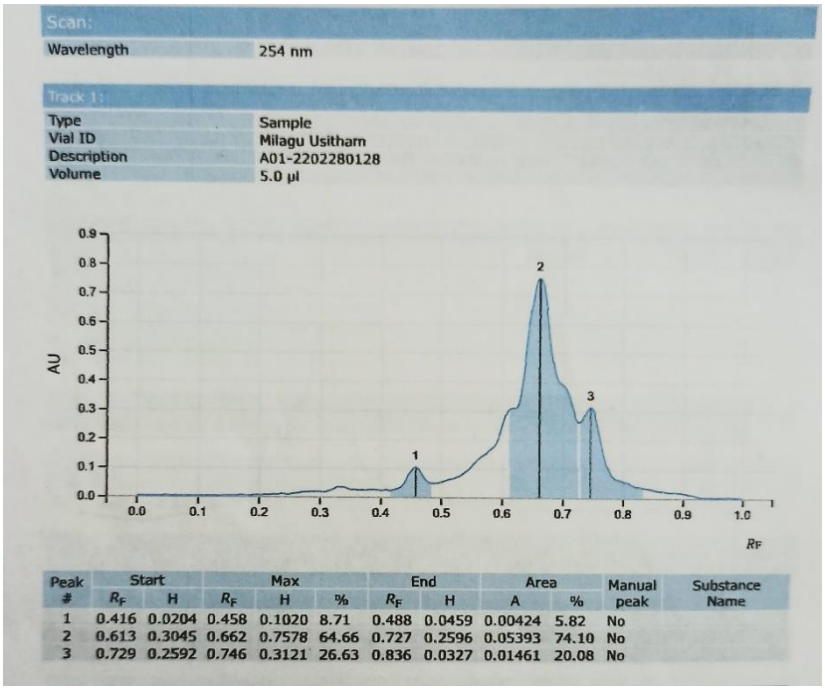
Chemical Analysis of *Milagu Usitham* –Miscellaneous

S.NO	Parameter	Result
1	Test for starch	Present
2	Test for reducing sugar	Absent
3	Test for alkaloids	Absent
4	Test for tannic acid	Absent
5	Test for unsaturated Compound	Absent
6	Test for amino acid	Absent

Table 11

Presence of carbonate and starch present in the sample of *Milagu Usitham*

HPTLC ANALYSIS

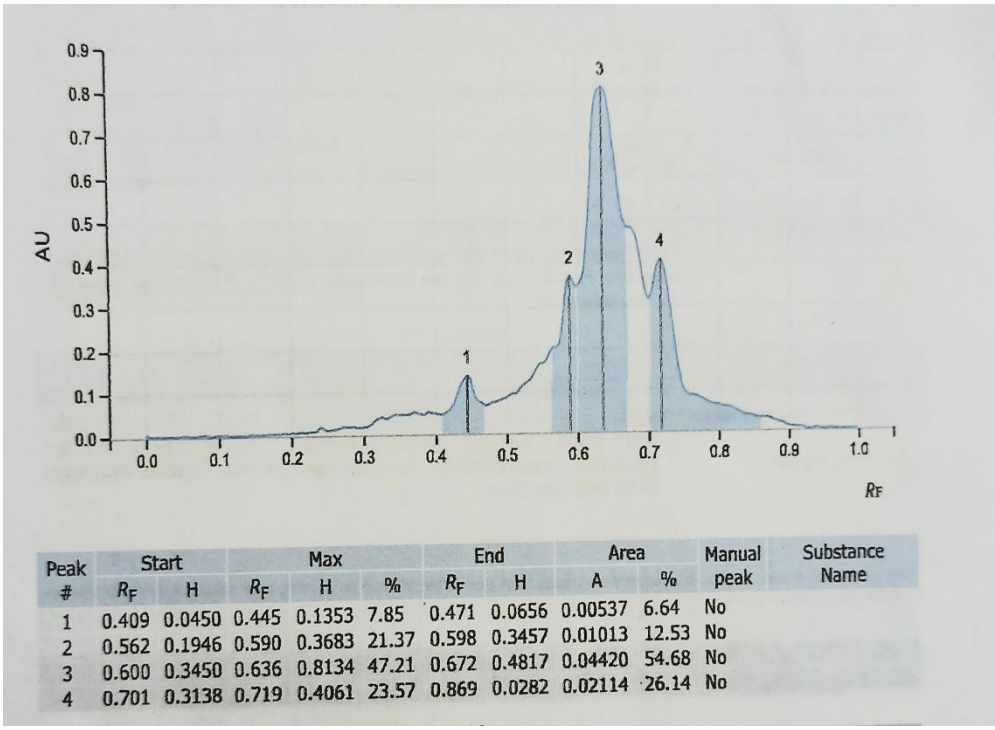


Peak #	Start R_F	Start H	Max R_F	Max H	Max %	End R_F	End H	Area A	Area %	Manual peak	Substance Name
1	0.416	0.0204	0.458	0.1020	8.71	0.488	0.0459	0.00424	5.82	No	
2	0.613	0.3045	0.662	0.7578	64.66	0.727	0.2596	0.05393	74.10	No	
3	0.729	0.2592	0.746	0.3121	26.63	0.836	0.0327	0.01461	20.08	No	

Figure 6

Peak	Start Rf	Start Ht	Max Rf	Max Ht	Max %	End Rf	End Ht	Area	Area %
1	0.416	0.0204	0.458	0.1020	8.71	0.488	0.0459	0.00424	5.82
2	0.613	0.3045	0.662	0.7578	64.66	0.727	0.2596	0.05393	74.10
3	0.729	0.2592	0.746	0.3121	26.63	0.836	0.0327	0.01461	20.08

Table no 12.1



Peak #	Start R_F	Start H	Max R_F	Max H	Max %	End R_F	End H	Area A	Area %	Manual peak	Substance Name
1	0.409	0.0450	0.445	0.1353	7.85	0.471	0.0656	0.00537	6.64	No	
2	0.562	0.1946	0.590	0.3683	21.37	0.598	0.3457	0.01013	12.53	No	
3	0.600	0.3450	0.636	0.8134	47.21	0.672	0.4817	0.04420	54.68	No	
4	0.701	0.3138	0.719	0.4061	23.57	0.869	0.0282	0.02114	26.14	No	

Figure 7

Peak	Start Rf	Start Ht	Max Rf	Max Ht	Max %	End Rf	End Ht	Area	Area %
1	0.409	0.0450	0.445	0.1353	7.85	0.471	0.0656	0.00537	6.64
2	0.562	0.1946	0.590	0.3683	21.37	0.598	0.3457	0.01013	12.53
3	0.600	0.3450	0.636	0.8134	47.21	0.672	0.4817	0.04420	54.68
4	0.701	0.3138	0.719	30.4061	23.57	0.869	0.0282	0.02114	26.14

Table no 12.2

Visualization at 254 nm

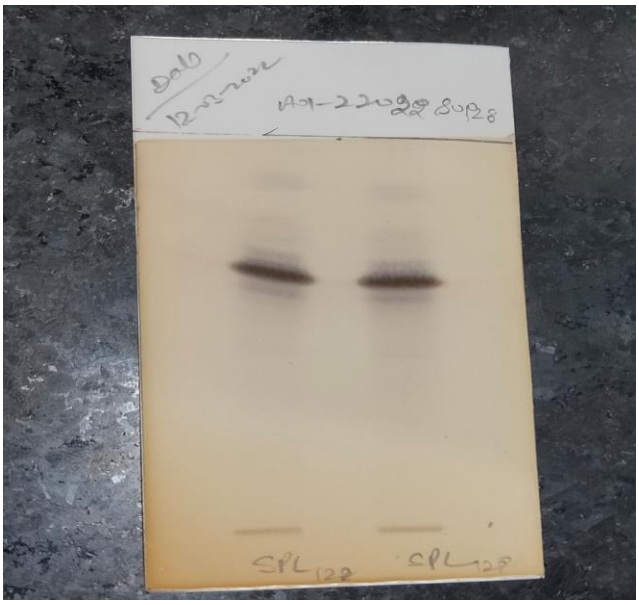


Figure 8.1

Dragendorff's derivatized TLC plate

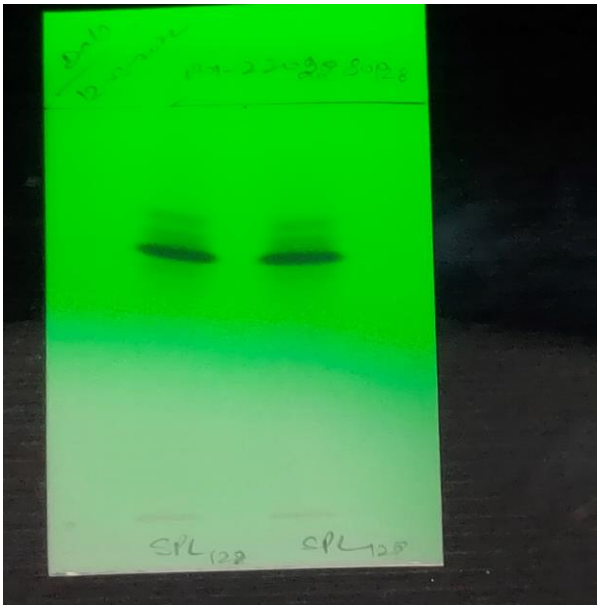


Figure 8.2

Results

HPTLC fingerprinting analysis of the sample reveals the presence of four prominent corresponds to presence of four versatile phytocomponents present with in it. Rf value of the peaks ranges from 0.409 to 0.701.

Hepatoprotective activity of *Milagu Usitham* against paracetamol induced hepatotoxicity in rats.

Effect of *Milagu Usitham* on SGOT, SGPT and ALP in paracetamol induced hepatotoxic rats

Groups	Treatment/Dose(p.o)	SGOT/AST (IU/L)	SGPT/ALT (IU/L)	ALP (IU/L)
Group I	Control group (honey)	71.20±0.05	42.20±2.10	165±6.30
Group II	Positive Control Paracetamol (1gm/kg b.wt)	133.90±1.80*	98.18±0.60*	436.00±8.40*
Group III	Standard group (Silymarin-50 mg/kg)+ Paracetamol	97.80±3.70**	77.80±0.40**	214±1.90**
Group IV	Test dose I MU(190 mg /kg b.wt) + Paracetamol	115.80±0.90**	83.50±1.30**	302.60±0.70**
Group V	Test dose II MU(760 mg/ kg b.wt) + Paracetamol	106.40±0.70**	82.43±1.20**	259±0.10**

Table 13.1

ANOVA followed by Dennett's test.*P<0.05,**P<0.01 and ***P<0.001 Vs Disease control.

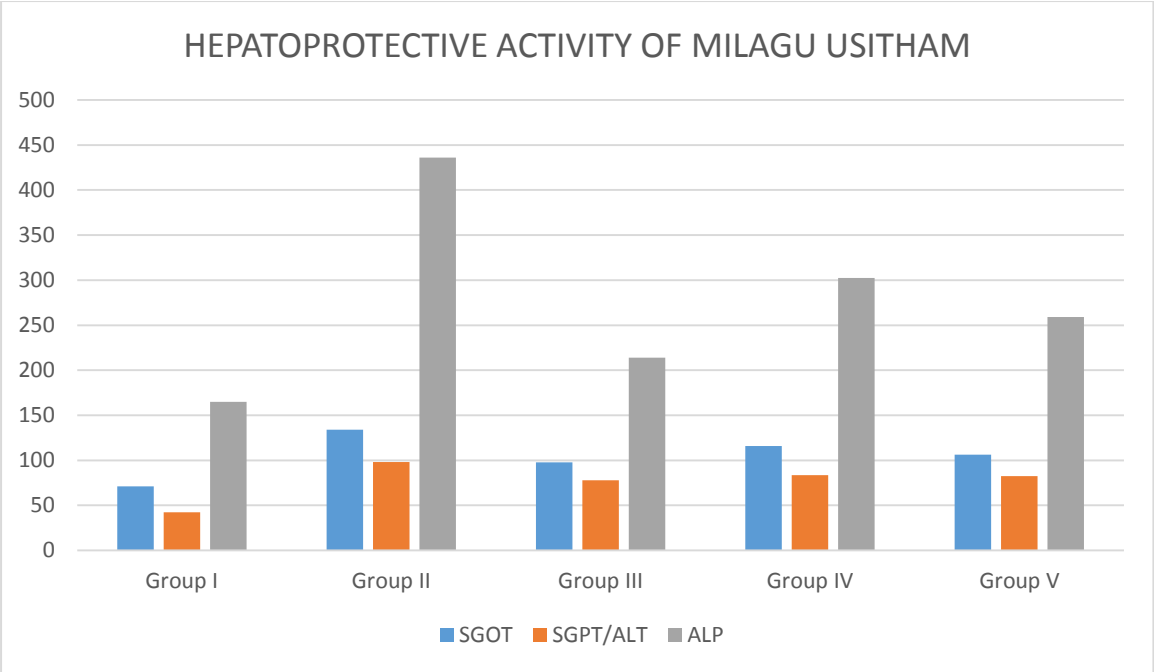


Figure 9.1

Effect of *Milagu Usitham* on Total Bilirubin and Total Protein in paracetamol induced hepatotoxic rats

Groups	Treatment/Dose(p.o)	TB(mg/dl)	TP(mg/dl)
GroupI	Control group (honey)	0.30±0.01	9.11±0.05
GroupII	Positive Control Paracetamol (1gm/kg b.wt)	0.77±0.01*	6.40±0.10*
GroupIII	Standard group (Silymarin-50 mg/kg)+ Paracetamol	0.51±0.07**	8.75±0.10**
GroupIV	Test dose I MU(90 mg /kg b.wt) +Paracetamol	0.63±0.03**	7.54±0.34**
GroupV	Test dose II Mu(760 mg/ kg b.wt))+ Paracetamol	0.58±0.01**	8.2±0.17*

Table 13.2 :

ANOVA followed by Dunnett‟s test.*P<0.05,**P<0.01 and ***P<0.001 Vs Disease control

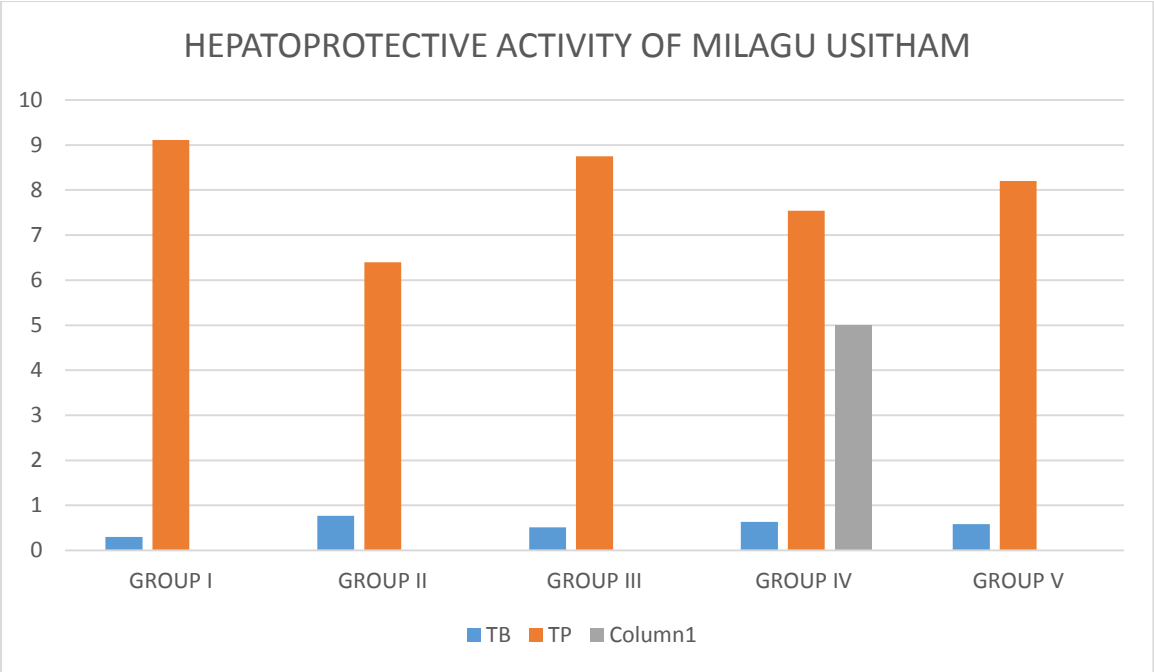
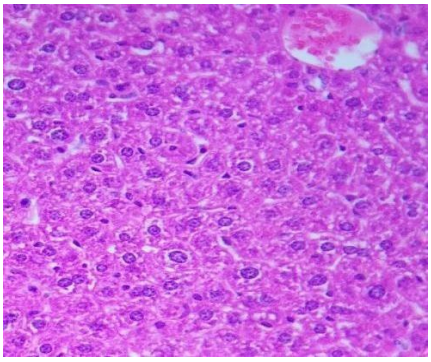
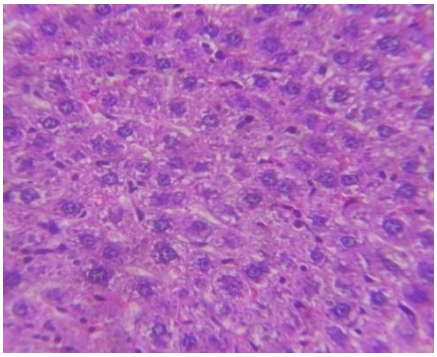


Figure 9.2

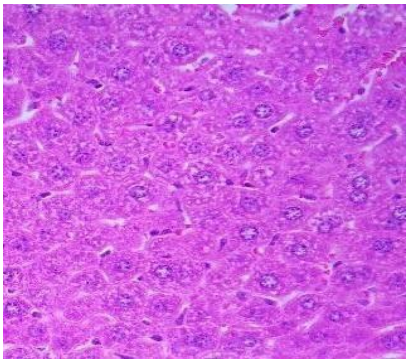
Histopathology report of liver



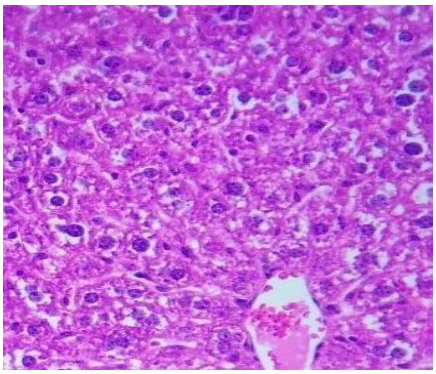
Control group figure no 10.1



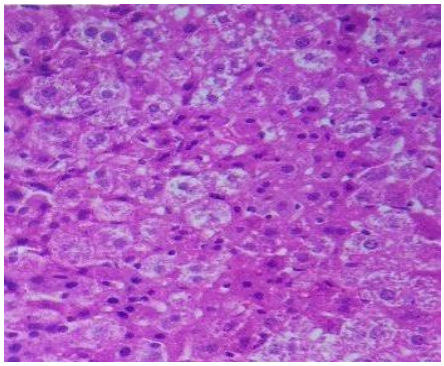
Disease control group figure no 10.2



Standard treated rats figure no 10.3



Milagu Usitham **group I figure no 10.4**



Milagu usitham **group II figure no 10.5**

Results

Group I : Control rats - Hepatic lobules with normal central vein were observed. (Figure 10.1)

Group II : Disease control - Extensive periportal degenerative changes were observed (figure 10.2)

Group III : Standard (Treated with silymarin) **-** Centrilobular zone appears normal with stable network of hepatocytes (figure 10.3)

Group IV: MU Dose I - Moderate derangement of hepatic parenchyma with mild congestion of central vein were observed. (Figure 10.4)

Group V: MU Dose II - Numerous hepatocytes appear with shrunken nucleus. No signs of nodular degeneration and cirrhosis (Figure 10.5)

Effect of MU on Urinary Parameters

(Urinary Vol, Diuretic Index, Percentage Urinary Excretion)

Control Group	**Urine pH**	**Urinary Output (ml)**	**Diuretic Index**	**Percentage Urinary Excretion**
Mean	6.008	1.24	-	29.72
Std. Deviation	0.4124	0.1369		3.198
Std. Error	0.1625	0.05536		1.32
Low Dose of MU	**Urine pH**	**Urinary Output (ml)**	**Diuretic Index**	**Percentage Urinary Excretion**
Mean	7.05	1.633	1.34	37.76
Std. Deviation	0.3257	0.1774		4.4325
Std. Error	0.1376	0.07503		1.70
Mid Dose of MU	**Urine pH**	**Urinary Output (ml)**	**Diuretic Index**	**Percentage Urinary Excretion**
Mean	7.05	1.837	1.55	39.97
Std. Deviation	0.3270	0.1858		4.467
Std. Error	0.1297	0.8312		1.723
High Dose of MU	**Urine pH**	**Urinary Output (ml)**	**Diuretic Index**	**Percentage Urinary Excretion**
Mean	7.04	2.128	1.69	51.2
Std. Deviation	0.3315	0.1751		4.712
Std. Error	0.1297	0.7291		1.775
Std Furosemide	**Urine pH**	**Urinary Output (ml)**	**Diuretic Index**	**Percentage Urinary Excretion**
Mean	7.57	3.525	2.87	83.85
Std. Deviation	0.3177	0.2678		6.754
Std. Error	0.1267	0.1247		2.8014

Table 14.1

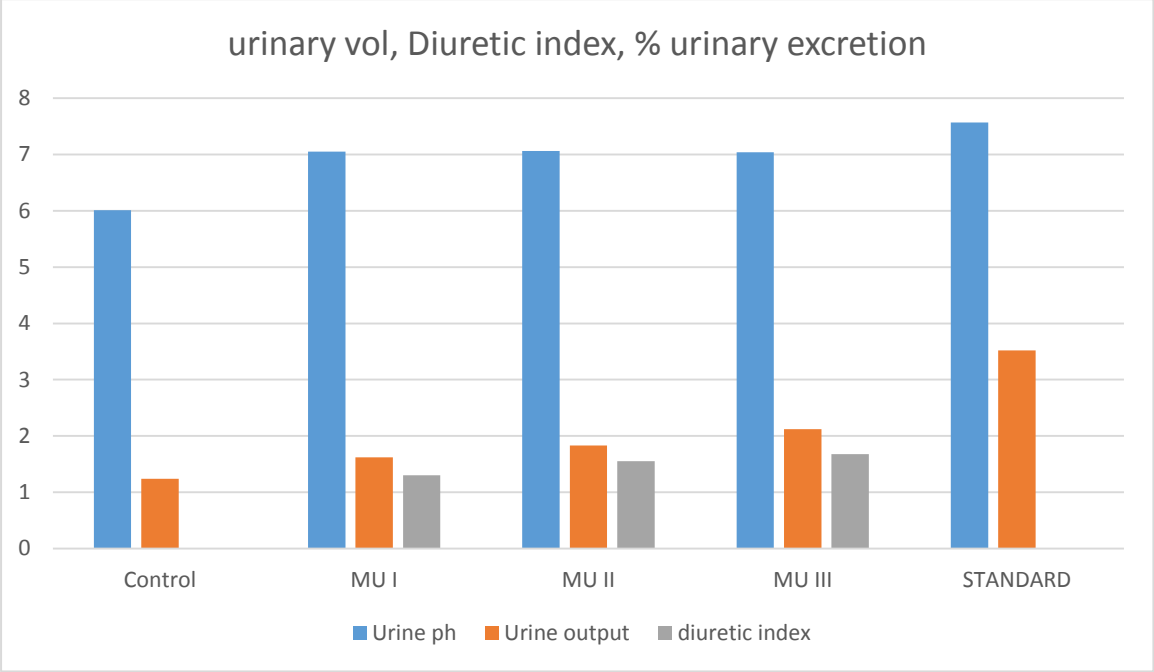

urinary vol, Diuretic index, % urinary excretion
8
7
6
5
4
3
2
1
0
Control
MU I
MU II
MU III
STANDARD
Urine ph
Urine output
diuretic index

Figure 11.1

Effect of MU on Urinary electrolyte excretion in rats

Control Group	**Na+ (Meq/L)**	**K+ (Meq/L)**	**Na+/K+**
Mean	14.43	53.29	0.258
Std. Deviation	3.07	3.757	0.1791
Std. Error	1.287	1.58	0.0355
Low Dose of MU	**Na+ (Meq/L)**	**K+ (Meq/L)**	**Na+/K+**
Mean	37.25	62.55	0.5707
Std. Deviation	4.18	3.315	0.05304
Std. Error	1.816	1.705	0.05275
Mid Dose of MU	**Na+ (Meq/L)**	**K+ (Meq/L)**	**Na+/K+**
Mean	44.51	66.34	0.6291
Std. Deviaation	7.08	3.452	0.1538
Std,Error	2.586	1.219	0.03714
High Dose of MU	**Na+ (Meq/L)**	**K+ (Meq/L)**	**Na+/K+**
Mean	53.17	68.09	0.7752
Std. Deviation	9.356	2.806	0.1373
Std. Error	3.412	1.257	0.07258
Std Furosemide	**Na+ (Meq/L)**	**K+ (Meq/L)**	**Na+/K+**
Mean	82.57	96.76	0.8753
Std. Deviation	5.429	5.325	0.01156
Std. Error	2.578	3.587	0.03587

Table 14.2

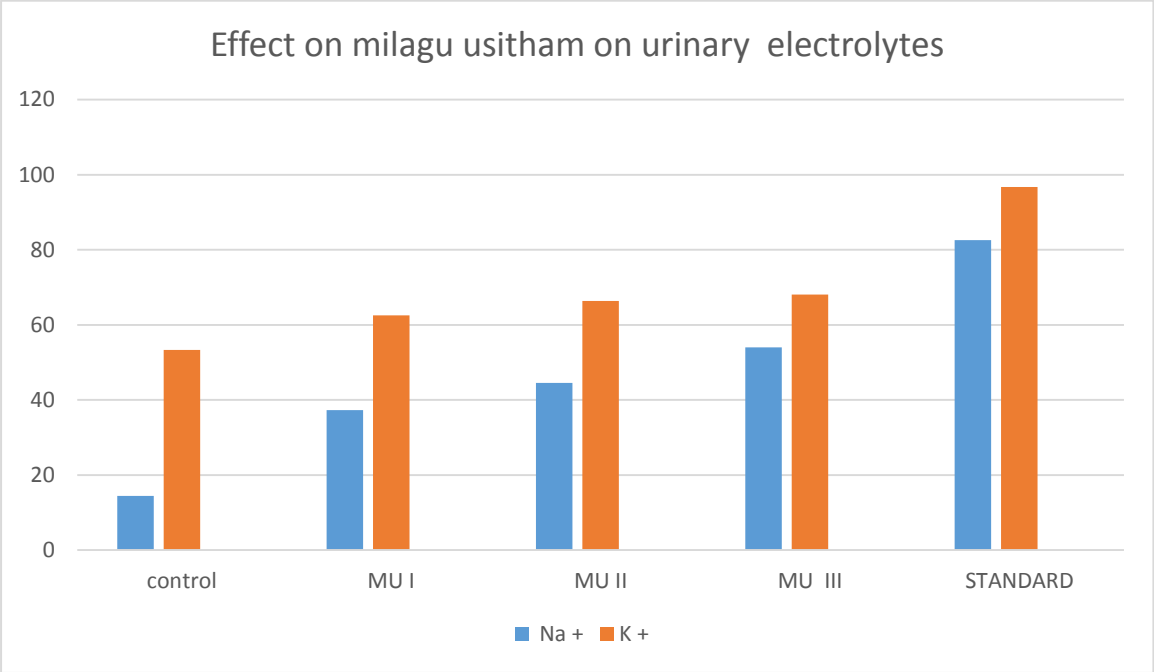


Figure 11.2

Trail drug of *Milagu Usitham* all the dose level has shown promosing diuretic activity By increasing the total urine output.The volume of urine output in *Milagu Usitham* 190 mg/kg treated group was 1.622 ± 0.07 ml urine of volume rats treated with *Milagu Usitham* 380 mg/kg was 1.837 ± 0.83 ml and urine of volume rats treated with *Milagu Usitham* 780 mg/kg was 2.128 ± 0.72 ml when the compared to that standard drug Furosemide (10 mg/kg,p.o) with the highest volume of 3.525 ± 0.12 ml The percentage of urine excretion in the control group rat was found 29.72 ± 1.32% while the trail drug *Milagu Usitham* treated group it was 37.76 ± 1.81 %, 39.97 ± 1.72 % and 51.2 ± 1.88 when compare to that of standard drug Furosemide with urine excretion 83.85 ± 2.70 there was significant increase in the diuretic index 1.86 in the high dose of test drug *Milagu Usitham* 780 mg/kg when compare to the group II rat with Diuretic Index of 1.34. Treatment with *Milagu Usitham* at all dose level has shown increased electrolyte (Na+ and K+) excretion in the urine. The concentration of urine Na+ and K+ in the high dose of *Milagu Usitham* 780 mg/dl treated group was found 53.17 ± 3.41Meq/L and 68.07 ± 1.22 which is significantly higher when compare the control group rats with Na+ (14.43 ±1.24 Meq /L) and K+ (53.27 ± 1.53 Meq /L) there were dose dependent increase in Na+ /K+ ratio in the *Milagu Usitham* treated group with the maximum of 0.77 ± 0.06 when compare to that of the standard drug Furosemide treated group with tha ratio of 0.87 ± 0.03.

Effect on anti oxidant activity

DPPH radical scavenging assay :

Effect of *Milagu Usitham* on
DPPH radical scavenging assay

Concentration (ug/ml)	%Inhibition of Milagu Usitham	%Inhibition of Ascorbic acid
10 µg/ml	16.98 ± 3.77	28.17 ± 2.304
20 µg/ml	27.2 ± 7.052	40.61 ± 2.653
40 µg/ml	32.64 ± 9.508	61.38 ± 2.406
60 µg/ml	44.63 ± 5.457	70.06 ± 2.105
80 µg/ml	54.92 ± 1.24	81.1 ± 4.19
100 ug/ml	63.08 ± 4.839	96.05 ± 0.7551

Data are given as Mean ± SD (n=3)

Table 15.1

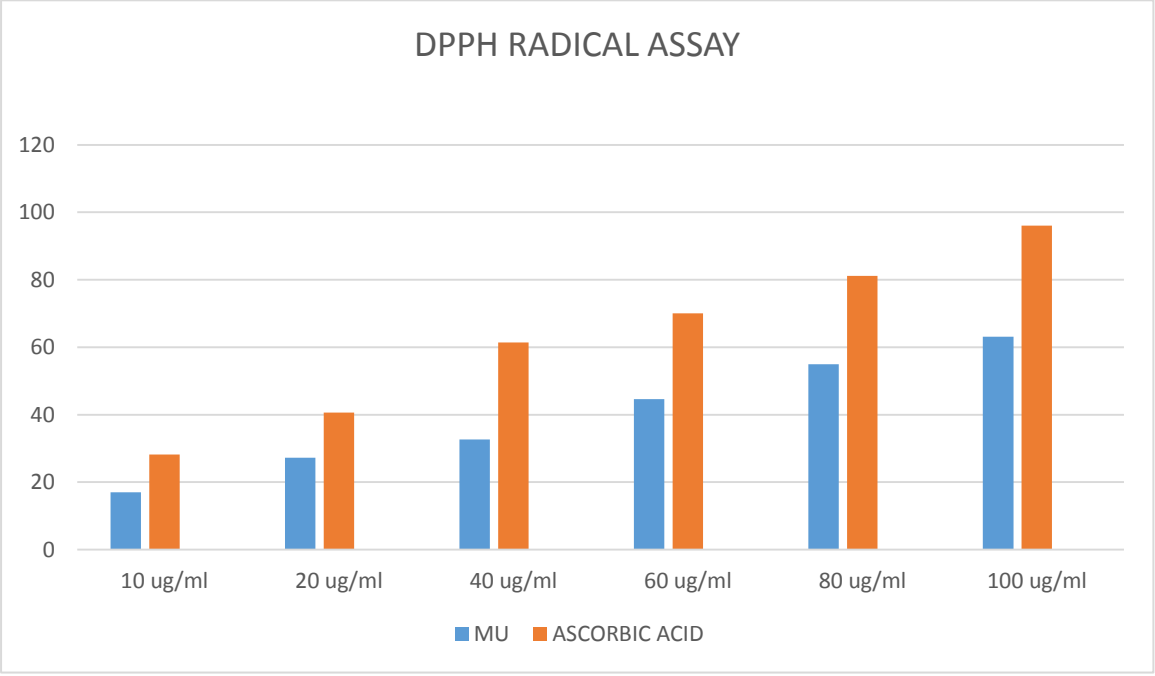


Figure 12.1

IC50 Values for DPPH radical scavenging Assay by *Milagu usitham* and Ascorbic Acid.

Test Drug / Standard	IC50 Value DPPH Assay ± SD (µg /ml)
Ascorbic Acid	33.65 ± 1.745
Milagu Usitham	71.57 ± 8.77

Data are given as Mean ± SD (n=3)

Table 15.2

Trial drug was screened for DPPH radical scavenging activity and the percentage of inhibition ranges from 16.98 ± 3.77 to 63.08 ± 4.839 % when compared with standard ascorbic acid with percentage inhibition of ranges from 28.17 ± 2.304 to 96.05 ± 0.7551 %. The IC50 value of the trial drug was found 71.57 ± 8.77 (µg /ml) when compared with standard ascorbic acid (IC_{50} value 33.65 ± 1.745µg/ml)

Nitric Oxide Radical Scavenging Assay

Effect of *Milagu Usitham* on Nitric Oxide radical scavenging assay

Concentration (ug/ml)	**%Inhibition of *Milagu Usitham***	**%Inhibition of Gallic acid**
10 µg/ml	3.802 ± 2.624	19.94 ± 2.331
20 µg/ml	9.263 ± 2.61	34.39 ± 2.964
40 µg/ml	13.36 ± 3.116	46.64 ± 1.501
60 µg/ml	18.48 ± 2.558	56.72 ± 3.55
80 µg/ml	25.99 ± 1.979	79.09 ± 2.135
100 ug/ml	31.44 ± 3.811	90.77 ± 1.471

Data are given as Mean ± SD (n=3)

Table 16.1

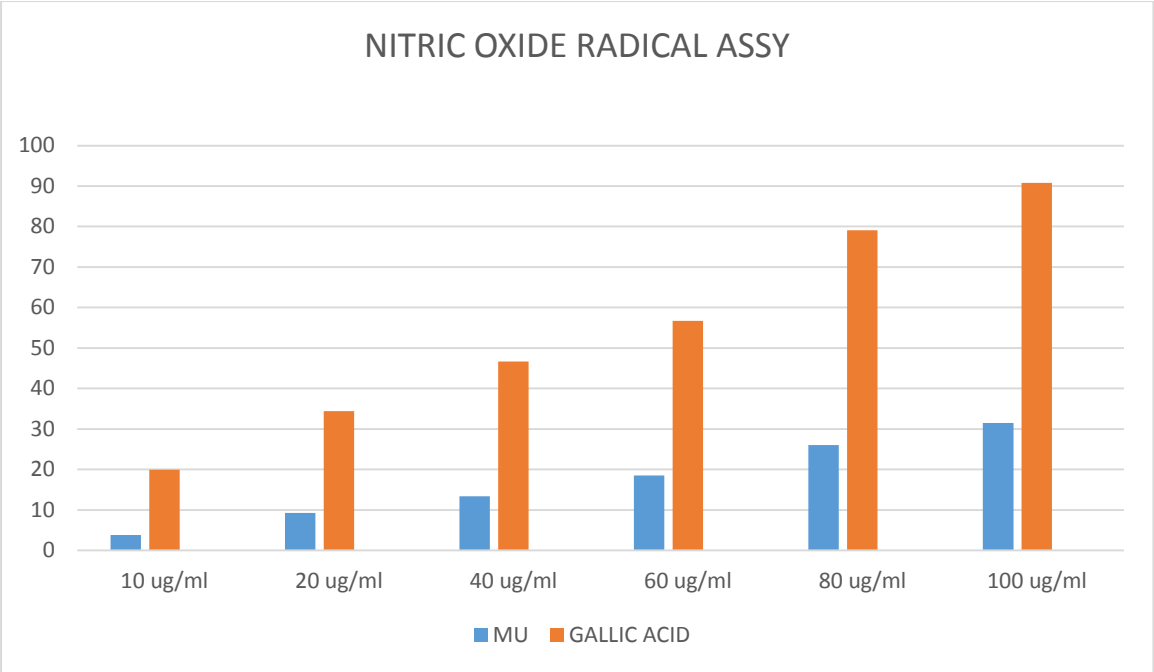


Figure 13

IC50 Values for Nitric Oxide radical scavenging assay by *Milagu Usitham* and Gallic Acid.

Test Drug / Standard	IC50 Value NO Assay ± SD (µg /ml)
Milagu Usitham	161.9 ± 10.05
Gallic Acid	45.7 ± 1.225

Data are given as Mean ± SD (n=3)

Table 16.2

NO radical scavenging activity of the trial drug revealed that the percentage inhibition of the test drug ranges from 3.802 ± 2.624 to 31.44 ± 3.811 % when compared with standard gallic acid with percentage of inhibition ranges from 19.94 ± 2.331 to 90.77 ± 1.471%. The corresponding IC50 value of the trial drug was found 161.9 ± 10.05 (µg /ml) when compared with standard gallic acid. (IC_{50} value 45.7 ± 1.225 µg/ml)

ABTS Radical Scavenging Assay

Effect of *Milagu Usitham* on ABTS radical scavenging assay

Concentration (ug/ml)	%Inhibition of Milagu Usitham	%Inhibition of Gallic acid
10 µg/ml	6.149 ± 1.084	21.37 ± 3.712
20 µg/ml	15.38 ± 2.755	44.69 ± 4.048
40 µg/ml	31.42 ± 2.479	58.11 ± 1.236
60 µg/ml	43.43 ± 2.341	73.25 ± 0.5839
80 µg/ml	55.52 ± 3.849	80.39 ± 1.601
100 ug/ml	68.63 ± 2.617	91.87 ± 0.4379

Data are given as Mean ± SD (n=3)

Table 17.1

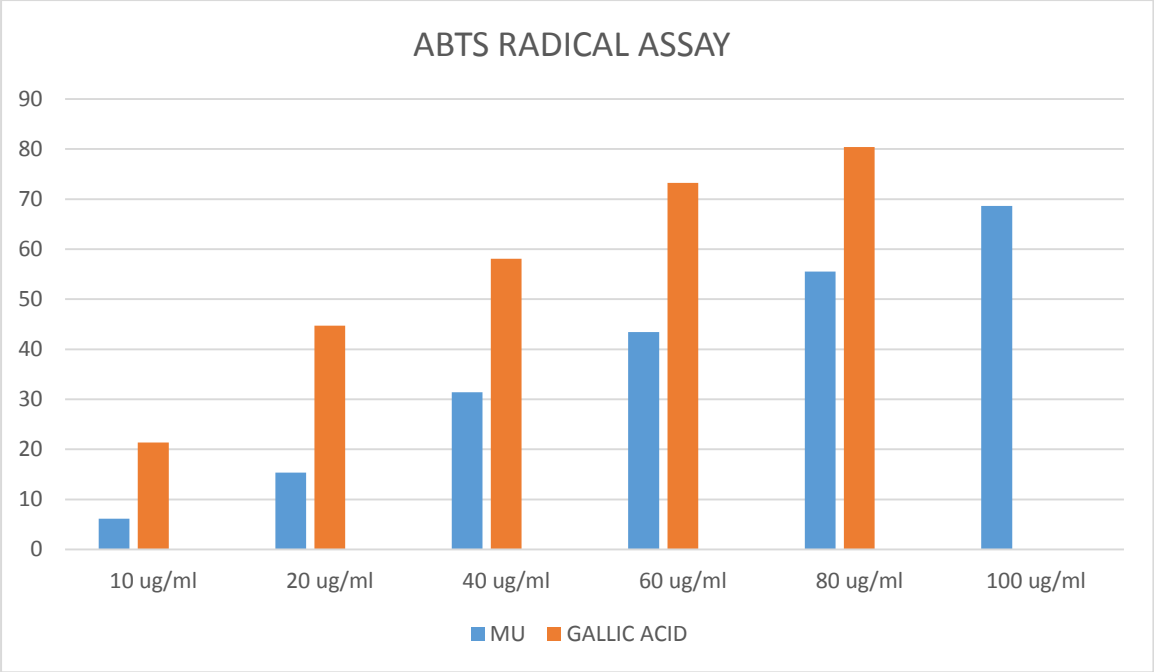


Figure 14

IC50 Values for ABTS radical scavenging assay by *Milagu usitham* and Gallic Acid

Test Drug / Standard	IC50 Value ABTS Assay ± SD (µg /ml)
Milagu Usitham	71.09 ± 2.997
Gallic Acid	35.35 ± 3.122

Data are given as Mean ± SD (n=3)

Table 17.2

Trial drug was screened for hydrogen peroxide radical scavenging activity and the percentage of inhibition ranges from 6.149 ± 1.084 to 68.63 ± 2.617 % when compared with standard gallic acid with percentage of inhibition ranges from 21.37± 3.712 to 91.87 ± 0.4379 % .The corresponding IC50 value of the trial drug was found to be 71.09 ± 2.997 (µg /ml) when compared with standard Gallic acid (IC_{50} value 35.35 ± 3.122 µg/ml)

Hydrogen peroxide radical scavenging assay

Effects of *Milagu Usitham* on Hydrogen peroxide radical scavenging assay

Concentration (ug/ml)	**%Inhibition of *Milagu Usitham***	**%Inhibition of BHA**
10 µg/ml	4.989 ± 2.461	29.39 ± 3.895
20 µg/ml	9.408 ± 3.308	39.79 ± 3.644
40 µg/ml	14 ± 4.036	53.61 ± 3.311
60 µg/ml	19.62 ± 3.787	56.73 ± 3.236
80 µg/ml	25.71 ± 2.246	73.36 ± 2.836
100 ug/ml	34.15 ± 5.905	92.15 ± 0.9974

Data are given as Mean ± SD (n=3)

Table 18.1

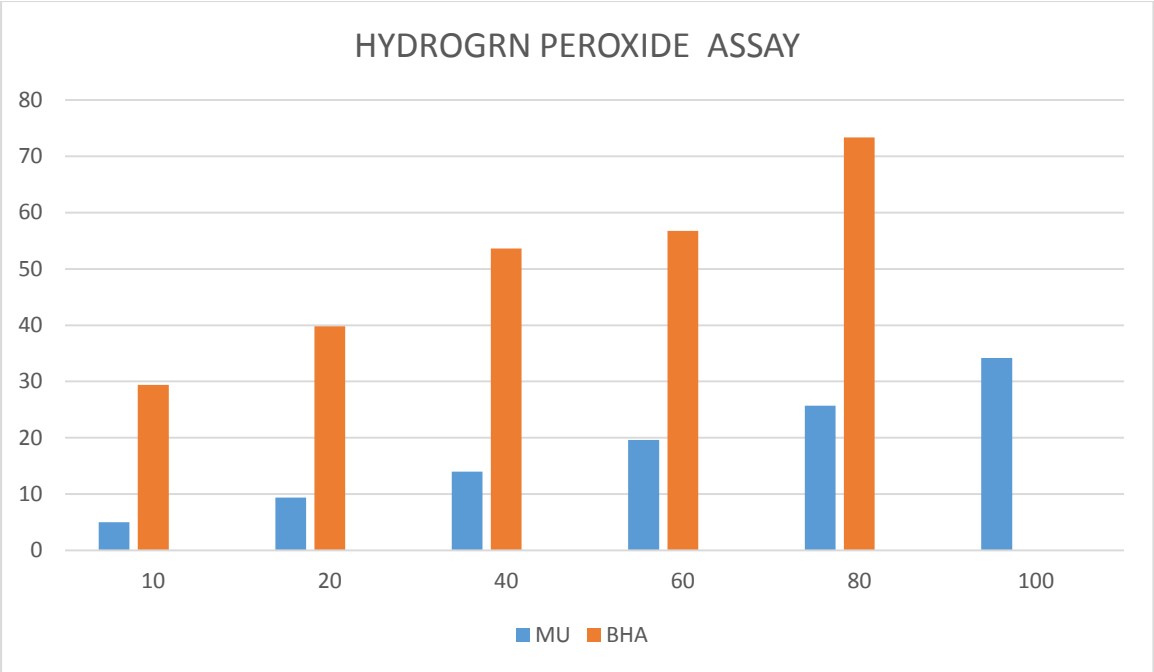


Figure 15

IC50 Values for Hydrogen peroxide radical scavenging assay by *Milagu Usitham* and Hydrogen Peroxide.

Test Drug / Standard	IC50 Value Hydrogen peroxide radical scavenging Assay ± SD (µg /ml)
Milagu Usitham	147.8 ± 29.3
BHA	39.79 ± 4.835

Data are given as Mean ± SD (n=3)

Table 18.2

Trial drug was screened for hydrogen peroxide radical scavenging activity and the percentage of inhibition ranges from 4.989 ± 2.461 to 34.15± 5.905 % when compared with standard BHA with percentage of inhibition ranges from 29.39± 3.895 to 92.15 ± 0.9974 %.The corresponding IC50 value of the trial drug was found 147.8 ± 29.3 (µg /ml) when compared with standard BHA (IC_{50} value 39.79 ± 4.835µg/ml).

Based on the results obtained from the In-vitro anti-oxidant assay for the sample *Milagu Usitham* it was concluded that the siddha formulation *Milagu Usitham* has promising anti-oxidant activity in the estimated assays

8. DISCUSSION

The drug *Milagu Usitham* was selected from the siddha literature *Anuboga vaidya navaneedham* Part – 9 for Standardization and pharmacological screening of Hepatoprotective, Diuretic in animal models and Anti Oxidants activities in Invitro model.

The ingredients of the test drug were identified and authenticated by Siddha experts. The drug was prepared as per the procedure and subjected to various studies such as qualitative, quantitative and pharmacological activities.

Literature review include drug review, which consist of both Siddha aspect, botanical aspect and scientific review have been done. This review was supported to the proposed study that supported the study

Drug review

Botanical aspect

The Drug review about the ingredients of *Milagu Usitham* from various text books was done. Botanical aspect explains the identification, description, active principle and medicinal uses of the plants. Siddha literatures related to the drug bring the evidence and importance of its utility in treating liver disorders and Ascities, In Gunapdam aspect *Milagu* indicates for curing jaundice (kamalai). *Poovarasu* indictes for curing ascities (peru vairu).

This review explained the preparation of *Chooranam* in detail including the purification of raw drugs, methods of preparation *Chooranam* and the Siddha parameters for the standardization of analyzing *Chooranam* The purification of drug like *Milagu* by the butter milk soaking in over night. The powdered of *Milgu Usitham* filtered through the white cloth so as to reduce the size of the particle in turn which enhances the bio-availability. The shelf life of the drug is improved by proper purification methods and preservation. Qualitative analysis includes organoleptic characters, chemical analysis and physicochemical properties of *Milagu Usitham.*

Analytical studies :

The drug *Milagu Usitham* is a fine powder .It is brown in color and solid in state. The total moisture present in the drug was established in loss on drying. Moisture content of the drug reveals the stability and its shelf-life. High moisture content can adversely affect the active ingredients of the drug. Thus low moisture content could get maximum stability and better shelf life The loss on drying value of *Milagu Usitham* is 11.52%.

pH it's a measure of hydrogen ion concentration and measure of acidic or alkalaine nature. The pH of the drug *Milagu Usitham* is 6.35 which is slightly acidic in nature and it's essential for its bioavailability and effectiveness.

The total ash value of the plant material indicated the amount of mineral and earthy material presents in the drug. The total ash value of *Milagu Usitham* is 2.21% which determine the absence of inorganic content.

The acid insoluble ash value of the drug denotes the amount of siliceous present in the plant. Its 0.73% for *Milagu Usitham*. These are indicating the approximate measure of chemical constituents of crude drug. These are indicating the approximate measure of chemical constituents of crude drug

Water-soluble extractive value plays an important role in evaluation of crude drugs and it is 3.07 % in *Milagu Usitham* **.**The alcohol-soluble extractive value was also indicative for the same purpose as the water-soluble extractive value and it is 7.86 % for *Milagu Usitham* **.**

In Chemical analysis**,** the drug *Milagu Usitham* revealed the presence of Carbonate and tannic acid .

HPTLC finger print :

The results from HPTLC finger print scanned at wave length 254 nm for chloroform extract of *Milagu Usitham.* It reveals the presence of four prominent corresponds to presence of four versatile phytocomponents present with in this *Milagu Usitham*. Rf value of the peaks ranges from 0.409 to 0.701

Pesticides residues analysis:

The results showed that there were no traces of pesticides residues such as Organochlorine, Organophosphorus, Organocarbamates and pyrethroids in *Milagu Usitham*.

Microbial Load:

The microbial load analysis confirms *Milagu Usitham* was free from microbial organisms and fungal infections.

Heavy Metal analysis by Atomic Absorption Spectrometry (AAS)

The result of heavy metals analysis of *Milagu Usitham* revealed the heavy metals like Lead, Arsenic, Cadmium and Mercury were found in range below the quantification limit (BQL)

Aflatoxin:

It indicates that the drug *Milagu Usitham* was free from Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, and Aflatoxin G2

Toxicity study

There was no mortality over a period of observation for 14 days in animals treated with a single dose of 2000 mg/kg. There were no other signs of toxicity and adverse effects. LD50 was considered to be more than 2000 mg/kg

Pharmacological Study

Hepatoprotective Activity

In the present study, elevated levels of SGOT, SGPT, ALP and Total bilirubin confirmed the paracetamol induced liver damage. The reactive oxygen species generation and lipid peroxidation of cell membranes leads to loss of membrane integrity, changes in membrane potential and an increase in membrane permeability ,which in turn results in leakage of enzymes from liver cells into circulation resulting in increased serum levels. On treatment with *Ka Milagu Usitham* **,** there was a reduction in serum enzyme levels which may be due to the reduction in the oxidative stress produced by paracetamol in toxication.

Due to the parenchymal damage of hepatic cells, there was a decrease in total protein levels of paracetamol treated groups. *Milagu Usitham* facilitated the regeneration of parenchyma of hepatic cells resulting in the significant increase of total protein levels in *Milagu Usitham* treated groups .

These data suggests a dose dependent Hepatoprotective activity of *Milagu Usitham.* This potency *Milagu Usitham* to reduce the elevated enzyme levels and raise the total protein levels is a clear manifestation of its hepatoprotectivity

Diuretic Activity

The diuretic activity of *Milagu Usitham* has been estimated using by Lipschitz test in Wistar albino rats at doses 190 mg/kg, 380 mg/kg and 760mg/kg. From the results it was concluded that administration of *Milagu Usitham* at the dose of 760 mg/kg exhibits significant diuretic activity.

Anti oxidant

Based on the results obtained from the Invitro anti-oxidant assay for the sample *Milagu Usitham* it was concluded that the Siddha formulation *Milagu Usitham* has promising anti-oxidant activity in the estimated assays

9. SUMMARY

- The test drug *Milagu Usitham* was selected from the *Siddha* literature *Anuboga vaidya navaneetham* to Standardize and pharmacological screening of hepatoprotective , diuretic and anti oxidant activities.

- All the ingredients were identified and authenticated by the experts and were purified and the medicine was prepared as mentioned in the Siddha literature.

- Reviews of literature in various categories were carried out. Siddha aspect, modern aspect and pharmacological aspect were disclosed about the ingredients of the drug *Milagu Usitham.*

- The drug was subjected to qualitative analysis such as physicochemical, chemical analysis, Instrumental and pharmacological analysis which provided the key ingredients present in the drug thus it accounts the efficacy of the drug

- Pharmacological studies revealed that the drug *milagu usitham* exhibited significant hepatoprotective, Diuretic activity in animal models and anti oxidant in Invitro model.

- Results and discussion gives the necessary justifications to prove the potency of the drug *Milagu Usitham.*

- Conclusion gives a compiled form of the study and explains the synergistic effect of all the key ingredients and activities that supports the study.

10. CONCLUSION

Milagu Usitham a traditional Siddha formulation was prepared as per the procedures mentioned in Siddha literature. From the literature evidence, Physico Chemical analysis, Chemical analysis, Instrumental analysis and Pharmacological studies, the author concludes that the drug *MilaguUusitham* is safe and it has significant effect of Hepatoprotective, Diuretic and Anti-oxidant activities.

It was concluded that the *Milagu Usitham* proved that it has remarkable medicinal value against the diseases of liver which is cost effective and easy to prepare. So this must be implicated in the future for clinical studies

11. REFERENCE

1. Dr. K. S. Uthamarayan, H. P. I. M., Siddha Maruthuvanga Surukkam, 2nd Edition, Department of Indian medicine and homeopathy, chennai, Reprint: 2010

2. Dr.T.Thirunarayanan, Introduction of Siddha medicine, Centre for traditional medicine and research (CTMR),Chennai, Pg.no :71,Reprint :2016

3. Hakkim B.Mohammed Abdullah Saheb ,Anuboga Vaithia Navaneetham Part:1,1975. Page no-66,67

4. Asrani S.K. Devarbhavi H, Eaton J, Kamath PS. Burden of liver diseases in the world. J Hepatol. 2019 Jan;70(1):151-171. doi: 10.1016/j.jhep.2018.09.014. Epub 2018 Sep 26. PMID: 30266282

5. Sarakku-Suthi Muraigal,Siddha Maruthuva Nool Veliyitu Pirivu,Indian Medicine and Homoeopathy dept., First edition, 2008

6. K.S Murugesa Mudhaliyar –Gunapadam Mooligai Vaguppu – Directorate of Indian Medicine and Homeopathy, Chennai – 106

7. Kannusamy pillai C, Sikitcha Rathna Deepam Ennum Vaidiya Nool, B.Rathina Nayakker and sons, Vol 1, 3rd edition, 1991

8. K.Radhakrishnan, AnubavaVaithiya DevaRagasiyam, B.R.Arangasamy,Chennai-79.

9. K.N.Kuppusamy Mudhaliar, K.S.Utthamarayan., Siddha Vaidhiya Thirattu, Department of Indian Medicine and Homoeopathy,Chennai. 2006

10. S.B. Ramachandran, yakobu vaithiyan chinthamani 700, first edition, thamarai noolakam ,1996

11.kannu samypillai, kannusamy parambarai vaithiyam , b. rathina nayakar and sons 5th edition , 2006

12. S.B.Ramachandran , agathiyar aayul vetham 1200, thamarai noolakam , 1999

13. S.B. Ramachandran , erana vaithiyam sinthamani , 1999.

14. S.mathrupotheswaran , agathiya vaithiya soothiram 650, narmatha pathipagam, 2010

15. Prema , agathiya vaithiya sinthamani venpa 4000, part-2 , 2nd edition, narmatha pathipakam, 2006.

16. Mohamad Abdulla Sahib, Kadukai Thani Maaanbu , Indian Medicine And Homoeopathy , 1992 .

17. Jeevaka Bharathi, Pokar Aruliya Vaithiya Saram 700, 3rd Edition , 2011

18. K.S. Murukaesa Mudhaliyar, Siddha Materia Medica (medicinal plants division), published by Indian medicine and homeopathy Dept, Chennai-600106

19. Mao QQ, Huang Z, Zhong XM, Xian YF, Ip SP. Piperine reverses the effects of corticosterone on behavior and hippocampal BDNF expression in mice. Neurochem Int. 2014 Jul;74:36-41

20. Somchai Sriwiriyajan, Aman Tedasen, Narissara Lailerd, Pleumjit Boonyaphiphat, Anupong Nitiruangjarat, Yan Deng, and Potchanapond Graidi . 2015 American Association for Cancer Research.st

21. Makhov P, Golovine K, Canter D, Kutikov A, Simhan J, et al. (2012) Co-administration of piperine and docetaxel results in improved anti-tumor efficacy via inhibition of CYP3A4 activity.

22. Selvendiran K, Sakthisekaran D (2004) Chemopreventive effect of piperine on modulating lipid peroxidation and membrane bound enzymes in benzo(a)pyrene induced lung carcinogenesis

23. Doucette CD, Hilchie AL, Liwski R, Hoskin DW (2013) Piperine, a dietary phytochemical,inhibits angiogenesis.

24. Gulten Okmen*, Mustafa Vurkun, Ali Arslan, Olcay Ceylan . lndian Journal of Pharmaceutical Education and Research | Vol 51 | Issue 3 | Jul-Sep, 2017(Special issue)

25. Xinpeng Bai,Weimin Zhang,Wenxue Chen,Wei Zong,Zhiyong Gua,and Xiaoqin Liu. African Journal of Biotechnology Vol. 10 (2), pp. 267-272, 10 January, 2011

26. Mao QQ, Huang Z, Zhong XM, Xian YF, Ip SP. Piperine reverses the effects of corticosterone on behavior and hippocampal BDNF expression in mice. Neurochem Int. 2014 Jul;74:36-41

27. Srinivasan K. Black pepper and its pungent principle-piperine: a review of diverse physiological effects. Crit Rev Food Sci Nutr. 2007;47(8):735-48

28. Platel K, Srinivasan K. Influence of dietary spices or their active principles on digestive enzymes of small intestinal mucosa in rats. Int J Food Sci Nutr. 1996 Jan;47(1):55-9

29. Platel K, Srinivasan K (2000) Influence of dietary spices and their active principles on pancreatic digestive enzymes in albino rats

30. sunila ES, Kuttan G (2004) Immunomodulatory and antitumor activity of Piper longum Linn. and piperine

31. R. Selvam, P. Kalaiselvi, A. Govindaraj, V.B. Alamurugan, A. Sathishkumar. Effect of *A. lanata* leaf extract and *Vediuppu chunnam* on the urinary risk factors of calcium oxalate urolithiasis during experimental hyperoxaluria. Pharmacological Research. 2001; 43(1): 89-93.

32. Niharika M, Harshitha V, Ashwini P, Srivinya B, Himabindhu J, Ramanjaneyulu K. Evaluation of In vitro anti urolithiatic activity of Chlorisbarbata. International Journal of Current Pharmaceutical Research. 2018; 10(3): 65-67.

33. Jain Monika, Bhandari Anil, Bhandari Aakanksha, Patel Priyanka. Isolation, Characterization and Invitro antiurolithiatic activity of Cerpegin alkaloid from *Ceropegia bulbosa* var. Lushii root. International Journal of Drug Development and Research. 2012; 4(4): 154-160.

34. . Kuppast, IJ, and Vasudev Naik, P. Diuretic activity of cardia dichotoma forester fruits. Indian Journal of Pharmaceutical Education and Research, 2005; 39: 186-187

35. Satoshkar RS, Bhandarkar SD, and Nirmal Rege N: Pharmacology and Pharmacotherapeutics, Popular Prakashan, Mumbai, 19th ed, 2005; 435-453.

36. Anonymous, the Wealth of India. Publication and information Directorate (CSIR), New Delhi, 1995; 223- 275.

37. Acharya SG, Momin AH and Gajjar AV (2012) Review of Piperine as A Bio-Enhancer. Am J Pharm Tech Res 2:32-44

38. K.kuppusamy muthaliyar, K. uthamarayan , Siddha Vaidya thirattu, indian medicine and homoeopathy , 2006

39. R. Thiyakarajan, Siddha maruthuvam sirappu, indian medicine and homoeopathy, 2013

40. Necheles, T.F., Boles, T.A., Allen, D.M., 1968. Glutathione peroxidase assay method. Journal of Pediatrics 72, 319.

41. Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in the auto oxidation of epinephrine and a simple assay for superoxide dismu- tase. Journal of Biological Chemistry 247, 3170–3185.

42. Ohkawa, H., Onishi, N., Yagi, K., 1979. Assay of lipid peroxidation in animal tissue by thiobarbituric acid reaction. Analytical Biochemistry 95, 351–354.

43. Doumas, B.T., Watson, W.A., Biggs, A.G., 1971. Estimation of total protein. Clinical Chemistry Acta 31, 87–96

44. Slater, T.F.(l 966). Nature (Lond .) **209:** 36

45. Sherlock, S., (1981). Biochemical Assessment of liver Function, Blackwell Scientific Publications, Oxford pp. 14-27

46. Velusamy , Theriyar Kudineer , Cental Council For Research In Ayurveda And Siddha 2nd Edition , 1996

47. Subramaniya Rathinam, Theraiyar Thaiala Varaka Surukam , 1944

48. R. Thiyarajan, Theriyar Tharu , 1997

49. Theva aashirvatham Samuel, Marunthu Sei Iyalum Kailaium , indian medicine and homoeopathy

50. kannusamy pillai , kannusamy parambarai vaidiyam , rathina nayakar and sond 2015

51.Kuzhathai Maruthuvam , K.Mueukesa Muthaliyar , Indian Medicine And Homeopathy ,2015

52. WHO guideline for assessing the quality of herbal medicines with reference to contaminants and residues. WHO Geneva. 2007

53. Luciana de CASTRO. Determining Aflatoxins B1, B2, G1 and G2 in Maize Using Florisil Clean Up with Thin Layer Chromatography and Visual and Densitometric Quantification. Ciênc. Tecnol. Aliment. vol.21 no.1 Campinas. 200

54. Pharmacopoeial Laboratory for Indian Medicine (PLIM) Guideline for standardization and evaluation of indian medicine which include drugs of Ayurveda, Unani and Siddha systems. Department AYUSH .Ministry of Health & Family Welfare, Govt. of India

55. Sethi PD. CBS Publishers and Distributors; 1996. HPTLC: High Performance Thin Layer Chromatography: Quantitative Analysis of Pharmaceutical Formulations.

56. Arup U, Ekman S, Lindblom L, Mattsson JE. High performance thin layer chromatography (HPTLC), an improved technique for screening lichen substances. *Lichenologist.* 1993;25:61–71.

57. Dayanand Reddy G, Bernard Mefi M, Bharathy H, Narasimha Kumar GV, Chitikela P Pullaiah, Ganesan R, Sathiya Rajeswaran P. Hepatoprotective Activity of Aruvadha churnam a Traditional Siddha Formulation against Paracetamol Induced Liver Damage in Rats. Research J. Pharm. and Tech 2018; 11(8): 3380- 3384. doi: 10.5958/0974-360X.2018.00622.4

58. Puckhaber L, Stipanovic R, Thespesenone and Dehydrooxoperezinone-6-methyl Ether, New Sesquiterpene Quinones from Thespesia populnea, Journal of Natural product.2004, 67:1571-1573

59. Vasudevan M, Gunnam K, Parle M, Antinociceptive and anti inflammatory effects of Thespesia populnea bark extract, Journal of Ethnopharmacology; 2007, 109:264-270

60. OECD (2000) Guidance Document on Acute Oral Toxicity. Environmental Health and Safety Monograph Series on Testing and Assessment No 24.

61. Patel, R.B. and Patel, M.R. and Patel, B.G. (2011) Experimental Aspects and Implementation of HPTLC. In: Shrivastava, M.M. HPTLC. New York: Springer, pp. 41- 54

62. P.Madhu kiran, A vijaya Raju, B ganga Rao, Investigation of hepatoprotective activity of *Cyathea gigantea* (Wall. ex. Hook.) leaves against paracetamol-induced hepatotoxicity in rats Asian pacific journal of tropical Bio medicine , (2012) 352- 356.

63. Eesha BR, Amberkar VM, Kumari KM, Sarath B, Vijay M, Lalit M,et al. Hepatoprotective activity of Terminalia paniculata against paracetamol induced hepatocellular damage in Wistar albino rats Asian Pac J Trop Med 2011; 4(6):466-469.

64. Galal RM, Zaki HF, Seif El-Nasr MM, Agha AM. Potential protective effect of honey against paracetamol-induced hepatotoxicity. Arch Iran Med. 2012; 15(11):674 – 680.

65. Lipschitz WL, Hadidian Z, Kerpcsar A. Bioassay of diuretics. J Pharmacol Exp Ther. 1943;79:97–110

66.Suresh babu , Christina , Tambi medabala, Praveen S Patel , Diuretic activity of ethonolic extract of leaves of *clissampelos pareira* in rats , Asian journal of pharmaceutical and clinical research vol 7, 2014

67. Abebaw Tegegne et.al ,evaluation of invivo diuretic activity of methanolic extracts of clutia abyssinica (Euphorbiacea)roots in wistar albino rats International Annals of Medicine vol1;1(8);2017

68. Jayasree T, Kiran KK. Evaluation of the diuretic effect of the chloroform extract of the Benincasa hispida rind (pericarp) extract in guinea pigs. J Clini Diagnos Res. 2011;5(3):578–82.

69. Dubey S, Verma Vijendra K, Sahu Amit K, Jain Amit K, Tiwari A. Evaluation of diuretic activity of aqueous and alcoholic rhizomes extracts of Costus speciosus linn in albino rats. Int J Res Ayur Pharm. 2010;1(2):648–52.

70. Badami, Omprakash, Dongr SH,Suresh B. *In-vitro* Antioxidant property of *Solanum Pseudocapsicum* leaf extract. *Indian J Pharmacol*.2005; 37:251-252

71. B. N. Panda, A. B. Raj, N. R. Shrivastava, and A. R. Prathani, "The evaluation of nitric oxide scavenging activity of Acalypha indica Linn Root," Asian Journal Research Chemistry, vol. 2, no. 2, pp. 148–150, 2009

72. N. Pellegrini, M. Ying, and C. Rice-Evans, "Screening of dietary carotenoids and carotenoid-rich fruits extract for antioxidant activities applying 2,2′-azobis (3-ethylbenzothine-6-surfonic acid) radical cation decolorization assay," Methods in Enzymology, vol. 299, pp. 384–389, 1999

73. Ruch R. J., Cheng S.-J., Klaunig J. E. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis. 1989;10(6):1003–1008

74 . WHO guideline for assessing the quality of herbal medicines with reference to contaminantsand residues WHO Geneva 2007

75.Lohar.D.R. Protocol For Testing of ASU medicines. Pharmacopoeial Laboratory for Indian Medicines Ministry of AYUSH.2007

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CERTIFICATE

Workshop on
Basic Research Techniques & Practices of Laboratory Animal Care

24 - 28 February, 2020

This is to certify that ..... **Dr. D. Velaman** .....

has participated as Trainee in the Workshop on Basic Research Techniques & Practices of Laboratory Animal Care on 24 - 28 February, 2020 at National Institute of Siddha, Chennai - 47.

Dr.V.Suba	*Dr.B.R.Senthilkumar*	*Prof.Dr.R.Meenakumari*
Organising Secretary	Co - Ordinator	Chairperson / Director

The Tamil Nadu Dr.M.G.R. Medical University

69, Anna Salai, Guindy, Chennai - 600 032.

This certificate is awarded to Dr. D.K.VELAMAN

for participating as Resource Person / Delegate in the 34th Workshop on

"How To Do a Good Dissertation & Publish? (Research Methodology and Biostatistics)"

(Virtual mode) for AYUSH Post - Graduates & Researchers organized by the

Department of Siddha, The Tamil Nadu Dr.M.G.R. Medical University

from 26 - 07 - 2021 to 30 - 07 - 2021.

Dr.N. KABILAN
PROFESSOR & HEAD, DEPT. OF SIDDHA

Dr.M.B. ASWATH NARAYANAN
REGISTRAR

Dr.SUDHA SESHAYYAN
VICE-CHANCELLOR

Institutional Animal Ethics Committee (IAEC)

NATIONAL INSTITUTE OF SIDDHA

(An autonomous body under Ministry of AYUSH, Govt. of India)
Tambaram Sanatorium, Chennai 6000 47.

CERTIFICATE

This is to certify that the project proposal No. **NIS/IAEC-22/R02/16112021/E7** entitled "Standardization and Pharmacological screening of Hepatoprotective, Diuretic and Anti - oxidant activities of siddha formulation *Milagu Usitham*" submitted by **Dr. D. Velaman** has been approved/ recommended by the IAEC of National Institute of Siddha in its meeting held on 16.11.2021 and **36 Rats (Male / Female)** have been sanctioned under this.

Authorized by	**Name**	**Signature /Date**
Chairperson	Prof. Dr. R. Meenakumari	
Main Nominee of CPCSEA	Prof. Dr. Geetha Ramesh	
Member Secretary	Dr. B.R. Senthilkumar	16. Nov 2021

TAMIL NADU VETERINARY AND ANIMAL SCIENCES UNIVERSITY

Laboratory Animal Medicine, Centre for Animal Health Studies, Chennai - 51

Laboratory Animals Request/ Reservation Form

Name of the Investigator : Dr. D. K. Velavan.
Designation : M.D., Ph Scholar, IIIrd year.
Department : Gunapadam,
Institution Name : National Institute of Siddha.
Postal Address : Tambaram Sanatorium, Chennai-47.

Phone / Mobile : 8190883082, 8072123011
Email ID : velgini97@gmail.com
CPCSEA approval Number : 1248/Go/re/S/09/CPCSEA

S.No	Species	Strain	No of animals required		Date Required	IAEC protocol Number* (MANDATORY)	Whether copy of IAEC approval attached? (MANDATORY)
			Male	Female			
1.	Rattus norvegicus	wistar albino rats	30	6	Last week of December 2021	NIS/IAEC-22/RO2/16112021/E7	Attached

(for price of animals, payment procedures, procedures for the receipt and transport of animal - please see http://www.tanuvas.tn.nic.in/lam.html)

Payment details:

DD No: _____ for rupees _____ drawn bank _____

(OR) Internet Banking (NEFT/ RTGS) transaction ID _____ transaction date _____

D. Velavan
Signature of Project Investigator / Guide
Date: 1/12/21
Place: Tambaram Sanatorium.

06/12/2021
Signature of HOD/ Institution with official seal
Date:
Place:

forwarded for action
A. [illegible]
1/12/2021

For Office Use Only

Form received on:
Application No:
Breeding required:
Animals Issued No:
Species: Male: Female: Total:

Payment details:
Entered in P.No. _____ of stock register
Vehicle No:

Signature and Date

APPLICATION FOR PERMISSION FOR ANIMAL EXPERIMENTS

Application to be submitted to the CPCSEA, New Delhi after approval of Institutional Animal Ethics Committee (IAEC)

Section -I

1. Name and address of establishment:

National Institute of Siddha
Tambaram Sanatorium
Chennai- 47

2. Registration number and date of registration:

1248/GO/Re/S/09/CPCSEA)
Date: 04/08/2020

3. Name, address and registration number of breeder from which animals acquired (or to be acquired) for experiments mentioned in parts B &C

1. The Tamilnadu Veterinary and animal Sciences University,
   Madhavaram milk colony,
   Chennai-600 051

2. Mass biotech
   127 thilaga nagar ,kanthalur village,
   Chengalpattu, Tamil nadu 603002
   CPCSEA Reg no: 2084/PO/Bt/S/19/CPCSEA

4. Place where the animals are presently kept (or proposed to be kept)

Animal House,
National Institute of Siddha
Tambaram Sanatorium, Chennai- 47

5. Place where the experiment is to be performed (Please provide CPCSEA Reg.No.)

Animal house,
National Institute of Siddha,
(1248/GO/Re/S/09/CPCSEA)

6. Date on which the experiment is to commence and duration of experiment

December 2021 to February 2022 & 3 months

7. Type of research involved (Basic Research/Educational/Regulatory)

Educational

Date: 01/11/2021
Place: Chennai -47

Signature
Dr.VELAMAN D
P.G Scholar III year (Investigator)
Department of Gunapadam